COMPARATIVE EVALUATION OF 'MTA' AND "PORTLAND CEMENT WITH THREE DIFFERENT RADIOPACIFYING AGENTS" ON OSTEOBLAST CELL SURVIVAL, ALKALINE PHOSPHATASE ACTIVITY AND GENETIC EXPRESSION OF MINERALIZATION - AN IN VITRO STUDY

A Dissertation submitted

in partial fulfilment of the requirements

for the degree of

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BRANCH – IV

CONSERVATIVE DENTISTRY AND ENDODONTICS

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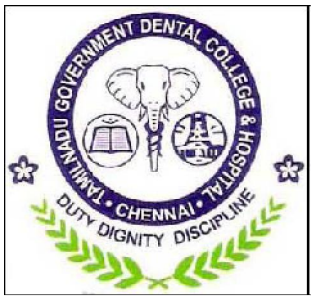
Certificate



This is to certify that **Dr. GOKUL.K,** Post Graduate student (2010 - 2013) in the Department of Conservative Dentistry and Endodontics, has done this dissertation titled "**Comparative Evaluation of 'MTA' and "Portland Cement with Three Different Radiopacifying Agents" on Osteoblast Cell Survival, Alkaline Phosphatase Activity and Genetic Expression of Mineralization – An In Vitro Study**" under my direct guidance and supervision in partial fulfillment of the regulations laid down by **The Tamil Nadu Dr. M.G.R. Medical University, Guindy, Chennai – 32** for **M.D.S. in Conservative Dentistry and Endodontics** (Branch IV) Degree Examination.

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DECLARATION

TITLE OF DISSERTATION	**Comparative Evaluation of 'MTA' and "Portland Cement with Three Different Radiopacifying Agents" on Osteoblast Cell Survival, Alkaline Phosphatase Activity and Genetic Expression of Mineralization – An In Vitro Study**
PLACE OF THE STUDY	**Tamil Nadu Government Dental College & Hospital, Chennai – 3.**
DURATION OF THE COURSE	**3 YEARS**
NAME OF THE GUIDE	**DR. M. KAVITHA**
HEAD OF THE DEPARTMENT	**DR. M. KAVITHA**

I hereby declare that no part of dissertation will be utilized for gaining financial assistance or any promotion without obtaining prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai – 3. In addition I declare that no part of this work will be published either in print or in electronic media without the guide who has been actively involved in dissertation. The author has the right to preserve for publish of the work solely with the prior permission of Principal, Tamil Nadu Government Dental College & Hospital, Chennai - 3.

Signature of the candidate **Head of the Department & Guide**

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This agreement herein after the "Agreement" is entered into on this day Dec 2012 between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, (hereafter referred to as, 'the college')

And

Mrs. Dr. M. Kavitha aged 42 years working as **Professor & HOD** in Department of Conservtive Dentistry & Endodontics at the college, having residence address at 69/4, Mettu street, Ayanavaram, Chennai – 23 (herein after referred to as the 'Principal Investigator')

And

Mrs. Dr. GOKUL.K aged 27 years currently studying as **Post Graduate student** in Department of Conservtive Dentistry & Endodontics, Tamilnadu Government Dental College and Hospital, Chennai - 3 (herein after referred to as the 'PG student and Co-investigator').

Whereas the PG student as part of his curriculum undertakes to research on **"Comparative Evaluation of 'MTA' and "Portland Cement with Three Different Radiopacifying Agents" on Osteoblast Cell Survival, Alkaline Phosphatase Activity and Genetic Expression of Mineralization – An In Vitro Study"** for which purpose the Principal Investigator shall act as principal investigator and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a Co-investigator

Whereas the parties, by this agreement have mutually agreed to the various issues including in particular the copyright and confidentiality issues that arise in this regard.

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6. All expenses pertaining to the research shall be decided upon by the Principal Investigator/Co-investigator or borne solely by the PG student. (co-investigator)

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8. The Principal Investigator shall suitably guide the Student Research right from selection of the Research Topic and Area till its completion. However the selection and conduct of research, topic and area of research by the student researcher under guidance from the Principal Investigator shall be subject to the prior approval, recommendations and comments of the Ethical Committee of the College constituted for this purpose.

9. It is agreed that as regards other aspects not covered under this agreement, but which pertain to the research undertaken by the PG student, under guidance from the Principal Investigator, the decision of the college shall be binding and final.

10. If any dispute arises as to the matters related or connected to this agreement herein, it shall be referred to arbitration in accordance with the provisions of the Arbitration and Conciliation Act, 1996.

In witness whereof the parties herein above mentioned have on this the day, month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

College represented by its **Principal** **PG Student**

Witnesses **Student Guide**

1.

2.

CONTENTS

LIST OF TABLES

LIST OF CHARTS

ABBREVIATIONS USED

ADA	***American Dental Association***
ALP	**Alkaline Phosphatase Assay**
ANOVA	**Analysis of Variance**
BA	***Bioaggregate***
BCA	**Bicinchoninic Acid**
BSP	**Gene Encoding for Bone Sialoprotein**
cDNA	**Complementary DNA**
COL I	**Gene Encoding for Collagen I**
DMEM	**Dulbecco'S Modified Eagle'S Medium**
DMSO	**Dimethyl Sulfoxide**
dNTPs.	**Deoxynucleotide Triphosphates**
EBA	**Ethoxybenzoic Acid**
EDTA	**Ethylenediaminetetraacetic Acid**
ELISA	**Enzyme-Linked Immunosorbent Assay**
FBS	**Fetal Bovine Serum**
FCS	**Fetal Calf Serum**
GADPH	**Gene Encoding for Glyceraldehyde 3-Phosphate Dehydrogenase**
HEMA	**Hydroxyethyl Methacrylate**
IL-1 α	***Interleukin-1 Alpha***
IL6	***Interleukin-6***
IRM	**Intermediate Restorative Material**
ISO	**International Organization for Standardization**
LPS	***Lipopolysaccharide***
MEM	**Minimal Essential Medium**
mRNA	**Messenger RNA**
MTA	**Mineral Trioxide Aggregate**
MTT	**3-(4, 5-Dimethyl Thiazol-2yl)-2, 5-Diphenyl Tetrazolium Bromide**
OCN	**Gene Encoding for Osteocalcin**
OD	**Optical Density**
OPN	**Gene Encoding for Osteopontin**
PBS	**Phosphate Buffered Saline**
PC	**Portland Cement**
qRT-PCR	**Quantitative Reverse Transcriptase - Polymerase Chain Reaction**
SD	**Standard Deviation**
TNF α	**Tumor Necrosis Factor Alpha**
TPVG	**Trypsin Phosphate Versene Glucose**
TUKEY HSD	***Tukey's* Honestly Significant Difference Test**
UV	***Ultraviolet***
WPC	**White Portland Cement**

ABSTRACT

AIM

To compare the Cytotoxicity, Alkaline phosphatase activity and Genetic expression of Mineralization between "White Portland Cement mixed with Radiopacifying agents (Iodoform/Zirconium dioxide/Bismuth oxide)" and "MTA" in Mouse MC3T3-E1 Osteoblast cells.

Materials & Methods

WPC was mixed with three different radiopacifying agents in the ratio of 4:1 by weight and divided into three groups. Along with MTA and control group, totally 5 groups were taken at a concentration of 0.02 mg/ml and compared the cytotoxicity by MTT assay and Alkaline phosphatase activity by Lowry method. Then qRT-PCR analysis was performed to detect the genetic expression of Mineralization and cytokines. LPS at a conc. of 10 µg/ml was added to the experimental materials and qRT-PCR analysis was again performed to detect the expression levels of cytokines despite LPS stimulation. The values were analyzed statistically by One Way Analysis of Variance followed by multiple comparison Tukey HSD test.

Results

The Cell survival ability was decreased in the 1^{st} and 2^{nd} day but significantly increased on the 3^{rd} day for all the experimental groups. The Alkaline phosphatase activity was decreased on the 3^{rd} day but increased gradually from 3^{rd} day to 15^{th} day for all the experimental groups. The gene expression of all Mineralization associated proteins were decreased on the 1^{st} and 2^{nd} day but significantly increased on the 3^{rd} day for all the experimental groups. With and without LPS stimulation, the cytokines, TNF α was detected at low levels, IL -6 was maintained and IL-1 α was highly suppressed for all the experimental groups. All the results were statistically insignificant among the experimental groups.

Conclusion

Hence it was concluded that Portland cement can be used in place of MTA after addition of any Radiopacifiers like Bismuth oxide, Iodoform and Zirconium dioxide.

Key words: Cytotoxicity, Gene Expression, MTA, Portland Cement.

Introduction

Pulpal diseases may progress to periapical lesions. The incidence of cysts & granulomas within periapical lesions are 55% & 70.07% respectively[6]. It is accepted that all inflammatory periapical lesions should be initially treated with conservative nonsurgical endodontic procedures. But in some cases, treatment failure is solved by endodontic surgery. Periapical surgery usually consists of apicocectomy, apical cavity preparation and root end filling to seal the communication pathways between the root canal system and periapical tissues.

For a long time, the root end filling materials of choice have been Amalgam, IRM, Super-EBA and Glass ionomer cements, Composites, Zinc phosphate, Poly HEMA, Biobond and EDH adhesive, Bone cements, Compomer, Hydroxyapatite cements, Resorcine-formalin resin, etc. However, these materials have the disadvantages of undergoing corrosion, electrolysis, delayed expansion and staining (amalgam), marginal leakage, moisture sensitivity and toxicity for vital tissues[41].

MTA (Pro Root MTA, Dentsply Tulsa, U.S.A.) basically composed of Portland cement 75% by weight, gypsum 5% by weight and bismuth oxide 20% by weight. The major component Portland cement is a mixture of dicalcium silicate, tricalcium silicate and tricalcium aluminate. Bismuth oxide is added to provide radiopacity greater than dentin.

MTA exhibits acceptable in vivo biologic performance when used for root-end fillings, perforation repairs, pulp capping, pulpotomy, and apexification treatment. MTA induces biomineralization of cementoblasts & osteoblasts and stimulate mineralization.

Portland cement is the most common type of cement in general use around the world. Type I Portland cement is the main component of MTA with addition of 20 wt% bismuth oxide at 4:1 ratio to provide radiopacity. Comparative chemical study and X-ray diffraction analysis of MTA and Portland cements proved that Portland cement is similar to MTA with the exception of Bismuth oxide which is present only in MTA[33, 25]. Histologic evaluation studies showed that Portland cement showed similar inflammatory results when compared with MTA. Portland cement also proved to be comparable with MTA in hard tissue formation when used as direct pulp capping material by maintaining pulp vitality[25, 4, and 55]. Portland cement does not have sufficient radiopacity to be visualised radiographically and thus a radiopacifying agent must be added to its composition. Bismuth oxide 20% is the radiopacifier present in MTA, atleast 15% of bismuth oxide is to be added to white Portland cement to provide sufficient radiopacity. However, it is questioned if bismuth oxide would be the best radiopacifying agent to be associated with Portland cement. **Coomaraswamy et al (2007)[14]** reported that the addition of bismuth oxide as a radiopacifier decreases mechanical stability by introducing flaws and increased porosity. Hence there is a need to search for an alternative radiopacifying agent to be associated with Portland cement.

The ISO 6876/2001 standard established that root canal sealers should be at least as radiopaque as 3mmAl. According to the American National Standards Institute and American Dental Association Specification No.57, endodontic filling materials should present a difference in radiopacity equivalent to at least 2mmAl in comparison to bone or dentin. Materials like Bismuth carbonate, Iodoform, Zirconium dioxide, Barium sulphate and Bismuth subnitrate had radiopacity values above that of dentin and the minimum recommended by the ANSI/ADA can be used

as radiopacifiers. The possible interference of the radiopacifiers with biocompatibility of Portland cement should be investigated. **Carlos Alberto et al (2006)[12]** reported that Iodoform 20wt% added with Portland cement showed similar tissue response as MTA, in a rat subcutaneous tissue implantation study.

An ideal root-end filling material should be biocompatible with normal tissues. Assessment of biocompatibility in vitro using cell culture techniques has been widely used. Cell types used varied from immortal cell lines to animal cells and fibroblasts. Primary osteoblasts have however been shown to be more appropriate for testing endodontic materials in cell culture as they are more sensitive and form mineralized nodules when exposed to differentiation media[43]. Enzyme assay measures the metabolic activity of cells grown over the materials under study. This can be done by using methyltetrazolium (MTT) assay. The MTT assay[40] is dependent on the intact activity of the mitochondrial enzyme, succinate dehydrogenase, which is impaired after exposure of cells to toxic surroundings.

The cellular events following an insult/injury in hard tissue formation generally undergo the following sequence; chemotaxis, proliferation, differentiation, mineralization of hard tissue matrix and cessation of hard tissue formation activity [47]. The presence of ALP is indicative of cells in differentiation phase. ALP is a hydrolytic enzyme with strong relationship to the process of mineralization and a known marker for hard tissue forming cells, osteonectin and osteopontin. To evaluate the effect of MTA and Portland cement on activities of osteoblast, expression of BSP, OPN, OCN, COL I genes were measured by the method of Quantitative Reverse Transcriptase Polymerized Chain Reaction (qRT-PCR) in the presence of those materials. This method is a technology to monitor gene expression over time, and it is

considered as the gold standard in the field of gene detection because of its sensitivity, precise gene quantification, real-time character, and time-saving characteristic in comparison to other methods such as Northern blotting, Southern blotting, and RNase protection assays.

MTA materials have demonstrated remarkable success as root-end fillings. Therefore it has been suggested that the cellular response to MTA may involve modulating inflammation. In 1997 and 1998**, Koh et al**[28, 29], reported that human MG-63 osteosarcoma cells produced IL-1alpha, IL-1beta, and IL-6 upon exposure to MTA. However in 1999, **Mitchell et al.**[35] found that the same cells produced only IL-6 in response to variant formulations of MTA, without expressing IL-1alpha.

Hence the aim of this study is to evaluate and compare 'MTA' and "Portland Cement with three different Radiopacifying agents (Iodoform, Zirconium oxide, Bismuth oxide)" by Osteoblast cell survival, Alkaline phosphatase activity and Genetic expression of Mineralization.

Aim & Objectives

The aim of this study was to compare the Cytotoxicity, Alkaline phosphatase activity and Genetic expression of Mineralization associated genes and Cytokines of White Portland Cement (WPC) (80wt%) mixed with (20wt%) Radiopacifying agents (Iodoform/Zirconium dioxide /Bismuth oxide) with MTA (Pro Root MTA) in Mouse MC3T3-E1 Osteoblast cells.

The objectives were:

1. To mix WPC 80wt% with Bismuth oxide 20wt%, WPC 80wt% with Iodoform 20wt%, and WPC 80wt% with 20wt% Zirconium dioxide.

2. To compare the cytotoxicity of above materials and MTA using MTT assay in mouse MC3T3-E1 osteoblast cells.

3. To compare the alkaline phosphates activity of above materials and MTA.

4. To compare the genetic expression of mineralization associated proteins of above materials and MTA using RT-PCR.

5. To compare the genetic expression of cytokines involved in the mineralization of above materials and MTA using RT-PCR with & without in vivo simulated inflammatory condition.

Review of Literature

Mineral Trioxide Aggregate (MTA):

Mineral Trioxide Aggregate (MTA) was a biomaterial that has been investigated for endodontic applications since the early 1990s. Originally developed by Torabinejad at Loma Linda university. MTA was first described in the dental scientific literature in 1993 and was given approval for endodontic use by the U.S. Food and Drug Administration in 1998.

Torabinejad et al (1995)[38] determined the chemical composition, pH, compressive strength and radiopacity of MTA. He showed that the main molecules present in MTA are calcium and phosphorus ions. In addition, MTA had a pH of 10.2 initially which then rised to 12.5 three hours after mixing. MTA was more radiopaque than Super EBA and IRM. MTA had a longer setting time of 2 hours and 45 minutes. At 24 hours MTA had the lower compressive strength of 40MPa but it increased after 21 days to 67 MPa.

Torabinejad et al (1995)[37] showed that the tissue reaction to MTA implantation in the mandible of guinea pig was milder than that observed with Super EBA implantation. It seemed that Super EBA and MTA were biocompatible.

Torabinejad et al (1995)[39] proved that MTA provided better adaptation seal than commonly used root end filling materials such as Amalgam, Super EBA and IRM.

Eng Tiong Koh et al (1998)[20] proved that the ELISA assays revealed raised levels of all interleukins at all periods when cells were grown in the presence of MTA; in contrast, cells grown alone or with IRM produced undetectable amounts. The macrophage colony stimulating factor was produced by cells irrespective of the

group. It seemed that MTA offers a biologically active substrate for bone cells and stimulated interleukin production.

Holland R et al (1999)[23] theorized that the tricalcium oxide in MTA reacted with tissue fluids to form calcium hydroxide, resulting in hard-tissue formation in a manner similar to that of calcium hydroxide.

Compared with calcium hydroxide, MTA had demonstrated a greater ability to maintain the integrity of pulp tissue. **Aeinehchi et al (2003)**[1] showed that histologic evaluation of pulpal tissue in animals and humans demonstrated that MTA produced a thicker dentinal bridge, less inflammation, less hyperemia and less pulpal necrosis compared with calcium hydroxide.MTA also appeared to induce the formation of a dentin bridge at a faster rate than did calcium hydroxide. The process by which MTA acted to induce dentin bridge formation, however, is not known.

In 2002, in addition to the traditional gray MTA (GMTA), White MTA (WMTA) was introduced. **Saeed Asgary et al (2005)**[48] concluded that concentrations of Al_2O_3 , MgO, and particularly FeO in WMTA was considerably lower than those found in GMTA. Differences in the observed FeO concentration were thought to be primarily responsible for the variation in color of the WMTA in comparison with GMTA.

Sarkar et al (2005)[51] showed that MTA materials were a mixture of a refined Portland cement and Bismuth oxide as radiopacifier and trace amounts of SiO_2, CaO, MgO, K_2SO_4, AND Na_2SO_4. The major component of Portland cement was a mixture of dicalcium silicate, tricalcium silicate, tricalcium aluminate, gypsum and tetracalcium aluminoferrite.

Camilleri (2006)[10] concluded that MTA materials formed a colloidal gel that solidifies to a hard structure in approximately 3-4 h. Hydrated MTA products had an initial pH of 10.2 which rises to 12.5 three hours after mixing. The setting process was described as a hydration reaction of tricalcium silicate and dicalcium silicate, similar to its parent compound Portland cement that needed sufficient water for reaction to occur.

MTA has a wide clinical application. **Peng et al(2006)**[42] showed that in primary molar teeth with vital pulp exposure caused by caries or trauma, a pulpotomy performed with MTA resulted in better clinically and radiographically observed outcomes. Fewer undesirable responses were recorded for MTA than when formocresol was used.

Ahmed et al (2008)[2] showed that Pro Root MTA has excellent sealing ability and could be used with or without matrix in repair of large furcation perforations and the use of IRM to repair large furcation perforations should be limited.

Witherspoon et al (2008)[62] showed that MTA obturation of canals with open apices was a viable alternative to the use of $Ca(OH)_2$ to induce apical closure.

William Saunders et al (2008)[61] **in** his prospective clinical study of periradicular surgery concluded that MTA as a root end filling material showed a high success rate of 88.8 %

MTA and Portland Cement

Jacob Saidon et al (2003)[26] compared the in vitro cytotoxic effect of MTA and Portland cement in L929 cells and tissue reactions of both the materials in bone

implantation in the mandibles of guinea pigs. There was no difference in cell reactions in vitro. Bone healing and minimal inflammatory response adjacent to ProRoot and Portland cement were observed, suggesting both materials were well tolerated. He concluded that MTA and Portland cement show comparative biocompatibility when evaluated in vitro and in vivo.

Razmi et al (2004)[44] evaluated the tissue reaction to implanted MTA and Portland cement in the mandible of cats. The physical and histological results observed with MTA were similar to those of Portland cement. Both the materials were considered biocompatible.

Renato Menezes et al (2004)[45] investigated the pulpal response of dogs' teeth after pulpotomy and direct pulp protection with MTA Angelus, ProRoot, Portland cement and WPC. All the materials demonstrated similar results when used as pulp capping materials. Pulp vitality was maintained in all specimens and the pulp had healed with a hard tissue bridge. The study concluded that Portland cement and MTA were equally effective as pulp protection materials following pulpotomy.

Durate et al (2005)[7] concluded that the release of arsenic from Portland cement and MTA were similar and were well below those considered to be harmful.

Islam et al (2005)[25] compared the major constituents present in ProRoot MTA, ProRoot MTA(tooth coloured) and ordinary Portland cement and white Portland cement using powder X-ray diffractometery. The main constituents were found to be tricalcium silicate, tricalcium aluminate, dicalcium silicate and tetracalcium aluminium ferrite in all the four cements with the additional presence of Bi_2O_3 in Pro Root MTA and Pro Root MTA (tooth coloured).

Daniel Araki Ribeiro (2005)[15] evaluated the genotoxic and cytotoxic effects of MTA and Portland cements in vitro using the alkaline single cell gel (comet) assay and trypan blue exclusion test, respectively on mouse lymphoma cells. The results demonstrated that the single cell gel assay failed to detect DNA damage after a treatment of cells by MTA and Portland cement. The study concluded that none of the compound tested were cytotoxic.

Marilia Gerhardt de Oliveira et al (2007)[33] analyzed and compared Portland cement with MTA. Similar chemical elements were found in all materials and there was a small percentile variation among them. Bismuth was detected only in MTA composition. In spite of the chemical similarity, it was observed that there was difference in the texture and in the particles of each material. Pro Root MTA presented the highest percentage of bismuth (9.2% on average). Except for bismuth, Portland cement and MTA presented similar chemical formulations.

De Deus et al (2007)[16] compared the sealing ability of four hydraulic cements, including Pro Root MTA and Portland cement. He concluded that no cement was capable of producing a fluid tight seal and the sealing ability promoted by MTA and Portland cement was similar.

Augusto Bodanezi et al (2008)[5] investigated the solubility of Mineral Trioxide Aggregate and Portland cement. Only Portland cement showed less than 3% weight loss through 24 hours. Detached MTA residues were heavier than those of Portland cement over the 3 to 168 hours. The study concluded that in an aqueous environment MTA was more soluble than Portland cement and exceeds the maximum weight loss considered acceptable by ISO 6876 (2001).

Bramante (2008)[8] analysed the concentration of arsenic in Portland cement and MTA. He concluded that the concentrations were well below the limit set in ISO 9917-1.

Amir Shayegan et al (2009)[3] compared the response of the pulp of primary pig teeth after capping with beta-tricalcium phosphate, white MTA, white Portland cement and calcium hydroxide. There was no significant difference between the materials in terms of primary pulp response, hard tissue formation and normal pulp tissue preservation. Beta-tricalcium phosphate, WMTA and White Portland cement in primary pig teeth were as effective as $Ca(OH)_2$ in pulp capping.

Taisa Regina Conti et al (2009)[55] reported two clinical cases in which Portland cement was applied as a medicament after pulpotomy of mandibular primary molars. At the 12 month follow up, clinical and radiographic examinations of the pulpotomized teeth and their periradicular area revealed that the treatments were successful in maintaining the teeth asymptomatic, preserving pulp vitality and formation of a dentin bridge immediately below the Portland cement.

Antonio Vinicius Holanda Barbosa et al (2009)[4] evaluated the short term response of human pulp tissue when directly capped with Portland cement. Portland cement exhibited some features of biocompatibility and capability of inducing mineral pulp response in short term evaluation. The results suggested that the Portland cement had a potential to be used as a less expensive pulp capping material in comparison to other pulp capping materials.

Radiopacifying Agents with Portland Cement

Coomaraswamy et al (2007)[14] investigated the effect of 0 to 10% bismuth oxide radiopacifier addition on the material properties of an endodontic Portland cement based system. The study concluded that the addition of Bi_2O_3 radiopacifier decreased mechanical stability by introducing flaws and increased porosity by leaving more unreacted water within the Portland cement. Flaws in the set cement matrix might exacerbate existing cracks ; moreover increased porosity is known to increase the solubility and thus the degradation of the material. This might potentially affect the longevity of the material, compared to that of pure Portland cement, because the set material was more likely to degrade and was thus more likely to be compromised as a sealant.

Camilleri (2007)[10] stated that the addition of bismuth oxide to MTA had been shown to affect the hydration mechanism of MTA. It forms part of the structure of calcium silicate hydrate, which was the main by product of cement hydration and also affects the precipitation of calcium hydroxide in the hydrated paste.

Marco Antonio Hungaro Durate et al (2008)[32] evaluated the radiopacity of Portland cement associated with the following radiopacifying agents: bismuth oxide, zinc oxide, lead oxide, bismuth subnitrate, bismuth carbonate, barium sulphate, iodoform, calcium tungstate and zirconium oxide. A ratio of 20% radiopacifier and 80% white Portland cement by weight was used for analysis. The study concluded that radiopacity of pure Portland cement was significantly lower than that of dentin. All the materials evaluated in the study had radiopacity values above that of dentin and the minimum recommended by ANSI/ADA.

Carlos Eduardo da Silveira Bueno et al (2009)[13] determined the ideal concentration of bismuth oxide in white Portland cement to provide it with sufficient radiopacity for use as an endodontic material (ADA specification #57). The readings of MTA and White Portland Cement with 15% bismuth oxide did not differ significantly from the reading observed for a thickness of 4mm of aluminium, which is considered ideal. White MTA and White Portland Cement with 15% bismuth oxide presented the radiopacity required for endodontic cement.

Saliba E et al (2009)[50] evaluated the strength and radiopacity of Portland cement with varying additions of bismuth oxide. He concluded that the addition of bismuth oxide did not seem to affect the compressive strength of Portland cement. All the bismuth oxide (10% to 30%) replaced cements had radiopacities higher than 3mm thickness of aluminium.

Yun Chan Hwang et al (2009)[66] compared the chemical constitution, radiopacity, and biocompatibility of Portland cement containing bismuth oxide with those of Portland cement and MTA. The chemical constitution was determined by energy-dispersive X ray analysis (EDX) attached to a scanning electron microscope. Cytotoxicity was evaluated using MTT assay. Tissue reaction was studied by subcutaneous implantation of the materials loaded in polyethylene tubes in the dorsal region of rats. The study concluded that the constitution of all materials were similar. However, the Portland cement were more irregular and had a larger particle size than MTA. The MTT assay revealed MTA to have slightly higher cell viability than the other materials. There was no significant difference in the tissue reaction between the experimental groups.

Camilleri (2010)[11] investigated the physical and chemical properties of Portland cement loaded with alternative radiopacifying materials (barium sulphate, gold and silver/tin alloy) for use as root end filling materials in a mineral trioxide aggregate like system. It was concluded that the bismuth oxide in MTA could be replaced by gold and silver/tin alloy. The physical, mechanical and chemical properties of the cement replaced with alternative radiopacifiers were similar and comparable to ProRoot MTA.

Cytotoxicity and Osteogenic Potential

Sema S. Hakki et al (2009)[52] investigated the effects of mineral trioxide aggregate (MTA) on survival, mineralization, and expression of mineralization-related genes of cementoblasts. MTT assay was performed to evaluate bioactive components released by MTA (0.002-20 mg/mL) on the cell survival. Gene transcripts for bone sialoprotein (BSP), OCN, collagen type I (COL I), and osteopontin (OPN) were evaluated by using qRT-PCR. They concluded that MTA did not have a negative effect on the cell survival and morphology of cementoblasts but MTA induce biomineralization of cementoblasts.

Yuan et al[65] (2010) investigated the cytotoxicity of bioaggregate (BA) and the effect of BA on mineral associated gene expression in osteoblast cells. The cytotoxicity of BA to mouse MC3T3-E1 osteoblast cells was evaluated via the MTT assay and the expression of mineral associated genes was assessed by qRT-PCR and compared with mineral trioxide aggregate (MTA). They found that the expression of collagen type 1, osteocalcin, and osteopontin genes significantly increased in the BA group compared with that in the MTA group on the second or third day of culture.

S. Rajan et al[47] (2008) determined in vitro assessment of MG-63 human osteosarcoma cells' alkaline phosphatase (ALP) activity when in contact with calcium hydroxide powder (CH), paste (CHP) and grey mineral trioxide aggregate (GMTA). BCIP-NBT assay was used and ALP activity quantified using ELISA reader at 410 nm. They concluded that all three materials exhibited increased ALP activity.

Deller-Quinn and Perinpanayagam[17] (2009) determined the attachment of osteoblasts to MTA surfaces and alteration in their expression of inflammatory cytokines by qRT-PCR. They found that Cells on MTA surfaces produced IL-6 but failed to express IL-1 despite LPS stimulation. They concluded that Osteoblast expression of inflammatory cytokines is altered on endodontic MTA surfaces.

Yan et al[63] (2010) investigated the cytotoxicity of bioaggregate to human periodontal ligament (PDL) fibroblasts and its effect on differentiation of human PDL fibroblasts and compared its performance to that of mineral trioxide aggregate. Cytotoxicity was assessed by MTT assay and gene expression of alkaline phosphatase (ALP) and collagen type I (COLI) was evaluated via qRT-PCR. They concluded that the Gene expression of COLI and ALP was induced by both BA and MTA compared to the control group.

Modareszadeh et al[36](2012) evaluated the cytotoxicity and alkaline phosphatase (ALP) activity of a new bioceramic root repair material, EndoSequence Root Repair Material and compared these characteristics with those of ProRoot MTA. Cytotoxocity was assessed by MTT assay and ALP activity of the cells was evaluated using a methylthiazol sulfophenyl assay. They concluded that EndoSequence Root Repair Material in general reduced the bioactivity and ALP activity of osteoblast cells whereas MTA had no effect on the cells.

Materials & Methods

Experimental Materials Used:

Pro Root MTA (Dentsply, U.S.)

Birla White cement (Grasim Ind Ltd. Aditya Birla group)

Bismuth Oxide LR (Chen Chemicals, India)

Iodoform (Vikash Pharma, India)

Zirconium dioxide (Lobal Ltd, India)

Armamentarium for Assessing Cyototoxicity

- Monolayer culture in log phase (MC3T3-E1)
- Minimal Essential Media **(**MEM) without 10% fetal calf serum (FCS)
- MTT reagent.
- TPVG reagent (100 ml)
    - PBS - 84mL
    - 2%trypsin -5mL
    - 0.2%EDTA -10mL
    - 10%glucose -500µL
    - Penicillin & streptomycin -500µL
- EDTA
- Fetal Calf Serum
- Dimethyl sulfoxide
- 0.4µ filter
- 5ml sterile storage vial
- Discarding jar, Tissue paper, spirit, cotton, marker pen and gloves
- Micropipette and tips

Armamentarium used for Alkaline Phosphatase Assay

- Monolayer culture bottle of MC3T3-E1 cell lines
- 5ml, 10ml, serological pipette
- Phosphate buffer saline (PBS)
- p- Nitrophenyl phosphate
- Sodium hydroxide
- UV Spectrophotometer (UV/VIS Digital spectrophotometer, Deep vision)

Armamentarium used for Genetic Studies

a) Chemicals used for qRT-PCR analysis.

Dulbecco‘s Modified Eagle‘s Medium (DMEM), Chloroform, Isopropanol, Agarose, Tris, Glycine, EDTA, Boric acid, Ethidium bromide, Trizol kit, Alizarin red, Cetylpyridinium, Oligonucleotide primers - Bone sialoprotein (BSP), Osteopontin (OPN), Osteocalcin (OCN), Collagen I (COL I), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Sigma, St. Louis, USA. Trypsin-EDTA solution, Sodium bicarbonate, Fetal bovine serum (FBS), Amphotericin B, Penicillin-Streptomycin were purchased from GIBCO-BRL, New York. Formalin and Ethanol were purchased from Sisco Research Laboratories (SRL) Pvt. Ltd, Mumbai, India. Gel loading dye was purchased from Geni, Banglore. Phosphate buffer saline (PH 7.4).

b) Armamentarium for qRT-PCR analysis.

Eppendorf tube, Cell Scraper, Thermal cycler (PCR machine), Electrophoresis Unit (Bio-Rad), Biofuge (Thermo), Microwave oven, Gel documentation system (Bio-Rad), UV visible spectrometer.

I. Methodology for Cytotoxicity

A.) Minimal Essential Media (MEM) Preparation:

The MEM vial is dissolved in the pre sterilized Millipore distilled water and mixed well, closed and sterilized at 15lbs at 121ºC for 15mins. Allow ingredients in the quantity, depending on the concentration of fetal calf serum (2% or 10%) mix well by shaking. Take care to avoid spills. Pass CO_2 using sterile pipette, shake the bottle, check pH and adjust to 7.2 to 7.4. The MEM bottles are kept for 2 days at 37ºC and checked for sterility, pH drop and floating particles. They are then transferred to the refrigerator (-20 C).

B.) Cell Culture:

Mouse osteoblastic Cells lines **MC3T3-E1** were obtained from the National centre for cell sciences, Pune. Cells were cultured in Minimal Essential Medium (MEM) supplemented with 10% heat inactivated Fetal bovine serum (FBS), 3% L- glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin (Hi media) grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

C.) Subculturing and Maintenance of Cell Line:

- The MEM Medium and TPVG which were maintained at -20^0 C were brought to room temperature for thawing.
- The tissue culture bottles were observed for growth, cell degeneration, pH and turbidity through an inverted microscope (100 X). After attaining 80% confluence of cells, sub culturing was done.

- The mouth of the bottle was wiped with cotton soaked in spirit to remove the adhering particles. The growth medium was discarded in a discarding jar.
- 4 – 5 ml of MEM without FCS was then added and gently rinsed with tilting. The dead cells and excess FCS were washed out and the medium was then discarded.
- TPVG was added over the cells and incubated at 37º C for 5 minutes for dissaggregation. The cells became individual and presented as suspension.
- 5ml of 10% MEM with FCS was added by using serological pipette. Gentle passaging was given by using serological pipette. (Process was repeated if any clumps were present.)
- After passaging, cells were splitted into 1:2, 1:3 ratios for cytotoxicity studies and were followed by plating method.

D.) Determination of Material Concentration

The Portland cement was mixed with three different Radiopacifying agents in 4:1 ratio by weight and grouped with MTA and Control as follows,

Experimental groups:

GROUP I - Control

GROUP II - MTA

GROUP III - White Portland Cement 80wt% + Iodoform 20wt%

GROUP IV - White Portland Cement 80wt% + Zirconium Dioxide 20wt%

GROUP V - White Portland Cement 80wt% + Bismuth Oxide 20wt%

- 0.5mg of each experimental material was dissolved in 4.5 ml of DMSO to give a working concentration of 10mg/ml. The working concentration was prepared fresh and filtered through 0.45 µfilter before each assay.
- 500µl of MEM without FCS was taken in 9 eppendorf tubes.
- Then 500µl of the working concentration (10 mg/ml) was added to the first eppendroff tube and mixed well. Then 500µl of this volume was transferred from first to last tube by serial dilution to obtain 10 desired different concentration of the each material (10 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.02 mg/ml, 0.01 mg/ml, and 0.002 mg/ml).
- As a result the volume remained constant but there was a change in concentration.

E.) Sampling:

Totally twenty 24 well culture plates were used. From those 20 plates, 2 plates were selected for each concentration of the experimental materials of 10 different concentrations (10 X 2). Two plates were further divided into 9 wells for each group of 4 groups (n=9, 4 groups, total 36 wells). Control group (Group I) was placed (n=9) in any one of the plates.

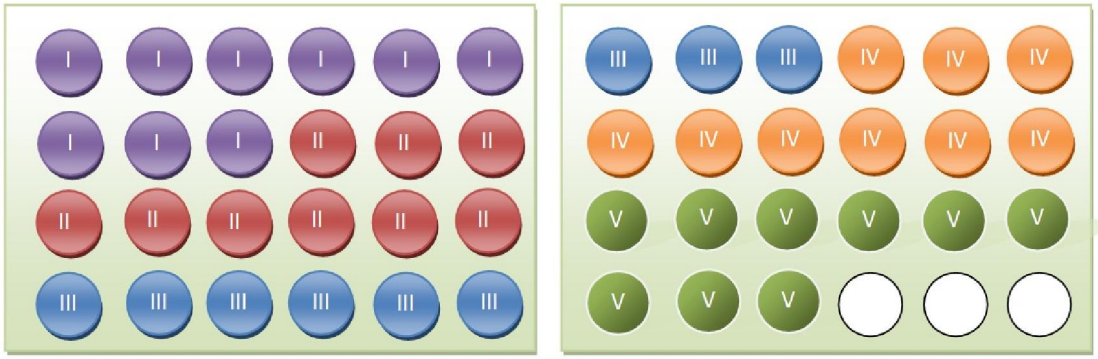
Ten different concentrations were 10 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.02 mg/ml, 0.01 mg/ml, and 0.002 mg/ml.

Schematic Illustration of Sampling

For example, 10 mg/ml concentration of sample materials were added into two 24 well plates in 9 wells for each group of 4 groups (n=9, 4 groups, 36 wells). Control

group was placed here (n=9) and was not placed in any other plates. The following pictures describe the method of sampling of 5 groups (Group I –V)

Figure 1: Two 24 well Culture plates



48hr monolayer culture of cells (MC3T3-E1) at a concentration of one lakh /ml /well seeded in all twenty 24 well culture plates. 1ml of medium (without FCS) containing defined concentration of the experimental material was added in twenty 24 wells so that each group at each specific concentration were placed in 9 wells (n=9). To the cell control wells (n=9) 1ml MEM without FCS was added. The plates were incubated at 37ºc in 5% CO2 environment and observed for cytotoxicity at 24, 48 and 72 hrs using MTT assay.

F.)MTT assay

MTT assay is a colorimetric assay that measures the reduction of 3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase enzyme. The MTT salt enters the cells and passes into the mitochondria where it is reduced to insoluble purple formazan product upon cleavage of the Tetrazolium by dehydrogenase enzyme. The cells were then solubilised with an

organic solvent and the pink solubilised formazan reagent was measured spectrophotometrically at 570 nm. Reduction of MTT can occur only in metabolically active cells and the level of activity is a measure of the viability and proliferation of the cells. Results were recorded as optical density (OD) units and a decrease in OD value denotes decrease in cell viability (i.e) increase in cytotoxicity.

II. Determination of Best Sample Material Concentration

The values were obtained for MTT assay for all ten concentrations and were recorded. The values were analyzed by cell viability percentage by using the formula {A/C * 100}, where A = OD values of experimental group and C=OD values of control group using Microsoft Excel 2010.

The concentrations of all experimental materials including 10, 2, 1, 0.5, 0.2, 0.1 and 0.05 mg/ml showed increased cell survival of osteoblasts from 24 to 72 hrs. But 0.02, 0.01 and 0.002 mg/mL concentrations of all experimental materials remarkably increased cell survival of osteoblasts (cell viability percentage of 80%) when compared with the other concentrations of MTA from 24 to 72 hrs. (This is consistent with results of **Sema S. Hakki et al. 2009** [50])

Hence the best concentration for all experimental materials was chosen as **0.02 mg/ml** (minimum dilution with best cell survival ability) and was used for alkaline phosphatase assay and qRT-PCR analysis.

III. Methodology for Alkaline Phosphatase (ALP) Assay

Osteoblast cells MC3T3E1 were grown to confluence and treated with different experimental material samples at a concentration of 0.02mg/ml. At defined time points, 3, 7 and 15 days, cells were harvested by scraping, rinsed twice with PBS and resuspended in lysis buffer (50 mM Tris, 100 mM glycine, 0.1% Triton X-100, 2.0 µg/ml aprotinin, 2.0 µg/ml leupeptin, 1.0 µg/mg pepstatin, pH 10.5). Cells were placed on ice, lysed by sonication and the lysate centrifuged at 15,000 rpm for 5 min at 4 ℃. Supernatants were then assayed for total protein content by the Bicinchoninic Acid (BCA) Assay and 30 µg of total protein was used to measure alkaline Phosphatase activity. Supernatants were incubated with substrate solution (3 mM p-nitrophenol phosphate, 0.7 M 2-amino-2-methyl-1-propanol, 6.7 mM $MgCl_2$, pH 10.3) for 30 min at 37 ℃ and then quenched by addition of 100 µL of 1N sodium hydroxide. Reactions were measured for optical density at 405 nm using a spectrophotometric plate reader.

IV. Quantitative Reverse Transcriptase - Polymerase Chain Reaction (qRT-PCR) Analysis of mRNA Expression

The experimental materials at a concentration of 0.02 mg.ml were added into two 24 well culture plates (total no of wells - 45, n = 9 for each group of 5 groups). Mouse osteoblast cells (MC3T3-E1) were then seeded at 1 lakh cells per well in DMEM with 10% FBS on the material mixed wells and cultured for 24, 48 and 72 hrs.

A.) Isolation of Total RNA

Total RNA was isolated from control and material treated cells by using total RNA isolation reagent (Trizol, Medox) kit following the method of Chomczynski and Sacchi (1987).

Principle

Single step guanidium acid phenol method emphasizes on the ability of guanidium isothiocyanate (GITC) to lyse tissues, denature protein and inactivate intracellular ribonuclease rapidly. The presence of β-mercaptoethanol in the mixture enhances the solubilization properties of the GITC extraction buffer. Acid phenol extraction (pH< 5.0) selectivity retains cellular DNA in the organic phase and aids in extraction of proteins and lipids. The addition of chloroform further removes lipids and establishes two distinct phases containing the DNA, proteins and lipids and an aqueous phase containing the RNA. The aqueous phase is separated and the RNA was precipitated by adding equal volume of isopropanol.

Reagents

Trizol kit has the following components:

- Phenol, guanidium isothiocyanate, urea, detergents, buffering agents and stabilizers.
- Chloroform (molecular biology grade).
- Isopropanol (molecular biology grade).
- 75% ethanol -To 7.5mL of absolute ethanol, 2.5mL of autoclaved deionised water.

Procedure

1 ml Trizol was added to control and material treated cells and swirled gently for 15 min and then kept at 4ºC for 5 min to permit complete dissociation of nucleoprotein complexes. To this 200 micro litre of chloroform was added, shaken vigorously for 15 seconds and placed on ice at 4ºC for 5 min. The lysate was then centrifuged at 12,000 rpm for 15 minutes at 4ºC, which yielded lower organic phase containing DNA and proteins and upper aqueous phase containing RNA. The volume of the aqueous phase was about 40-50% of the total volume of the lysate.

The aqueous phase was carefully transferred to a fresh eppendorf micro centrifuge tube without disturbing the interphase. Equal volume of isopropanol was added, mixed and kept at 4ºC for 10 min. It was again centrifuged at 12,000 rpm for 10 minutes at 4ºC to precipitate the RNA.

The supernatant was removed and the pellet was washed twice with 75% ethanol and air dried and centrifuged at 7500 rpm for 5 minutes. The RNA pellet was then dissolved with 25 µl of sterile deionised water and placed in a water bath at 60ºC for 10 minutes to ensure maximum solubility of RNA. The RNA sample was subsequently vortexed gently and quantified before storing at -80ºC.

B.) Quantification of RNA

Diluted RNA sample was quantified spectrophotometrically by measuring the absorbance (A) at 260 nm. An absorbance of 1OD is equivalent to RNA concentration of 40 g/ml. Therefore, the yield can be calculated by multiplying the absorbance at 260 nm with dilution factor and 40. The purity of RNA preparations were assessed by

determining the ratio of absorbance of sample at 260 nm and 280 nm. The purity of RNA obtained was ~1.6-1.8.

C.) Reverse Transcriptase-Polymerase Chain Reaction

The reverse transcriptase-polymerase chain reaction (RT-PCR) involves the conversion of mRNA present in the total RNA into cDNA and then amplifies a specific region of interest present in the cDNA. Reverse transcriptase-polymerase chain reaction of BSP, OPN, OCN, COL I, TNF-α, IL-6, IL-1α, and GAPDH were performed using Qiagen two step RT-PCR kit. This enzyme reverse transcriptase catalyzes the conversion of mRNA into cDNA. Reverse transcriptase polymerase chain reaction was done using a two step kit in which the reverse transcription reaction and the amplification can be carried out separately.

Principle

RT-PCR selectively amplifies the first strand of cDNA that has been synthesized *in vitro* from mRNA templates by reverse transcription. The cDNA is first denatured by heating in the presence of two oligonucleotide primers and four dNTPs. The reaction mixture is then cooled to allow the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerases.

Reverse Transcription

2µg of total RNA isolated control and experimental groups were subjected to reverse transcription using Qiagen kit, Germany. The components include ,

- Volume of buffer : 2 µl
- dNTP : 2 µl
- Oligo dT(10 µM) : 2 µl
- RT enzyme : 1 µ1
- RNA : Variable (2µg)
- Water : Variable

- Total : 20 µl

Polymerase chain reaction

Description

PCR Master mix includes Nuclease-free water and PCR Master mix, 2X. PCR Master mix is a premixed, ready to use solution containing Taq DNA polymerase dNTPs, $MgCl_2$ and reaction buffers at optimal concentrations for efficient amplification of DNA templates by PCR.

PCR Master Mix, 2X

50 units/ml of Taq DNA polymerase supplied in a proprietary reaction buffer (pH 8.5), 400µM dATP, 400µM dCTP, 400µM dTTP, 400µM dGTP, 3mM $MgCl_2$.

General Reaction Protocol

The components in the PCR include

Component	Volume	Final Conc.
2 X PCR master mix	12.5µl	1X
Upstream Primer (6µM)	2.5 µl	0.6 µM
Downstream Primer (6µM)	2.5 µl	0.6 µM
Upstream Primer(GAPDH) (6µM)	1 µl	0.25µM
Downstream Primer(GAPDH) (6µM)	1 µl	0.25µM
Template DNA	1 µl	1 in 10 dilution
Water	4.5µl	-
Total Volume	25 µl	-

The reaction mix was centrifuged in a microcentrifuge for 10 seconds and placed in a thermal cycler. PCR was performed using standard procedures (3-step Cycling). The PCR products were separated using agarose gel electrophoresis and visualized and documented using quantity one software (Bio Rad, USA).

Oligonucleotide primers

For amplification of the target genes, the following primers were used.

S.NO	PRIMERS
1.	**BSP** Forward 5 '- CAGGGAGGCAGTGACTCTTC-3' Reverse 5'-AGTGTGGAAAGTGTGGCGTT-3'
2.	**OPN** Forward 5 '-GTCAAGCAGGAGTGCAATCG-3' Reverse 5'- GTCAAGCAGGAGTGCAATCG-3'
3.	**OCN** Forward 5 '-CTGACAAAGCCTTCATGTCCAA-3' Reverse 5'- GCGCCGGAGTCTGTTCACTA-3'
4.	**COL I** Forward 5'-CCTGGTAAAGATGGTGCC-3' Reverse 5'-CACCAGGTTCACCTTTCGCACC-3'
5.	**TNF-α** Forward 5 '-CCACCACGCTCTTCTGTCTAC-3' Reverse 5'- AGGGTCTGGGCCATAGAACT-3'
6.	**IL-1α** Forward 5 '-TTGACAAACAAATTCGGTACA--3' Reverse 5'- GAGGTGCCCATGCTACA-3'
7.	**IL-6** Forward 5 '-TTGACAAACAAATTCGGTACA--3' Reverse 5'- GAGGTGCCCATGCTACA-3'
8.	**GAPDH** Forward 5 '-AGGTCGGTGTGAACGGAT TTG-3' Reverse 5'- TGTAGACCATGTAGTTGAGGTCA-3'

D.) Agarose Gel Electrophoresis

Agarose Gel Electrophoresis is an effective method for the identification of DNA molecules **(Sambrook *et al.*, 1989).**

Principle

The generated cDNA fragments were resolved in 2% agarose gel under an applied electric field. DNA molecules migrate towards the anode due to negatively charged phosphate along the backbone of DNA. The rate of migration of linear DNA is inversely proportional to its molecular weight. Thus, the larger molecules travel at a much lower speed when compared to smaller one.

Reagents

- TBE buffer 1X: (Tris, Boric acid, EDTA) (pH 8.0): 5.4 g tris, 2.75 g boric acid and 370mg of EDTA were dissolved in 500 ml of autoclaved RNAse and DNAse free water and the pH was adjusted to 8.2.
- 1% Ethidium bromide (EtBr) in RNAse and DNAse free water
- 2% Agarose in 1 X TBE buffer: 1g of agarose was transferred to a conical flask containing 50ml of 1 X TBE buffer, melted in a oven to ensure complete solubility.
- Gel loading dye (6 X)

  The gel loading dye (6 X) was procured commercially in ready to use form.

Procedure

1 g of agarose was added to 50 ml 1 X TBE buffer (2%). It was then melted in a microwave oven, the volume was made up to 50 ml with TBE buffer and 2 µl of 1% EtBr was added, evenly mixed and cooled to 40ºC. It was then poured into a sealed gel-casting platform and comb was inserted after ensuring the absence of air bubbles. The gel was then allowed to harden. After 45 minutes, the comb was removed taking

care that the sample wells were not disturbed. The platform was then immersed in the tank with electrophoresis buffer.

5 µl of PCR products from each reaction tube was mixed with 2 µl of 6X gel loading dye and loaded to each well. A 100 bp molecular weight marker DNA was simultaneously loaded in the first lane to check the specificity of the product. The power supply was turned on and the current adjusted to 80-100 mA, for 2 hours, and then the resolved cDNA fragments in the gel were visualized and documented using Quantity one software (Bio-Rad, USA). The band intensity of resolved cDNA fragments of BSP, OPN, OCN, TNF-α, IL-1α, IL-6 were normalized with that of the internal control GAPDH and expressed as OD units relative to GAPDH.

E.) Stimulation with Bacterial LPS

Experimental Groups

GROUP I - Control

GROUP II - LPS alone

GROUP III - LPS + MTA

GROUP IV - LPS + White Portland Cement 80wt% + Iodoform 20wt%

GROUP V - LPS + White Portland Cement 80wt% + Bismuth Oxide 20wt%

GROUP VI - LPS + White Portland Cement 80wt% + Bismuth Oxide 20wt%

Mouse MC3T3-E1 preosteoblasts (100,000 cells per well) were seeded onto 0.02 mg/ml of sample mixed three 24-well tissue culture plates (n=9, 6 groups & 54 wells). After 6 hours of growth, fresh medium was added containing 10 micro g/mL LPS in all the groups except group I which serves as a control. The LPS were

obtained from *Escherichia coli.* After 24, 48 and 72 hrs hours of incubation, replicate cultures were harvested for RNA extraction and reverse-transcription polymerase chain reaction (RT-PCR) analysis was performed as mentioned above.

Statistical Analysis

The values were recorded for each group. The values were analyzed statistically by One Way Analysis of Variance followed by multiple comparison Tukey HSD test. All statistical analyses were done using SPSS 11.5. Statistical significance was determined at $P < 0.05$.

FLOW CHART

Procedural flowchart for assessing Cytotoxicity

MTT ASSAY

The MC3T3-E1cells were plated in twenty 24 well plates at a concentration of One lakh /ml /well.

↓

24 h after plating, cells were washed twice with 100 µl of MEM and grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

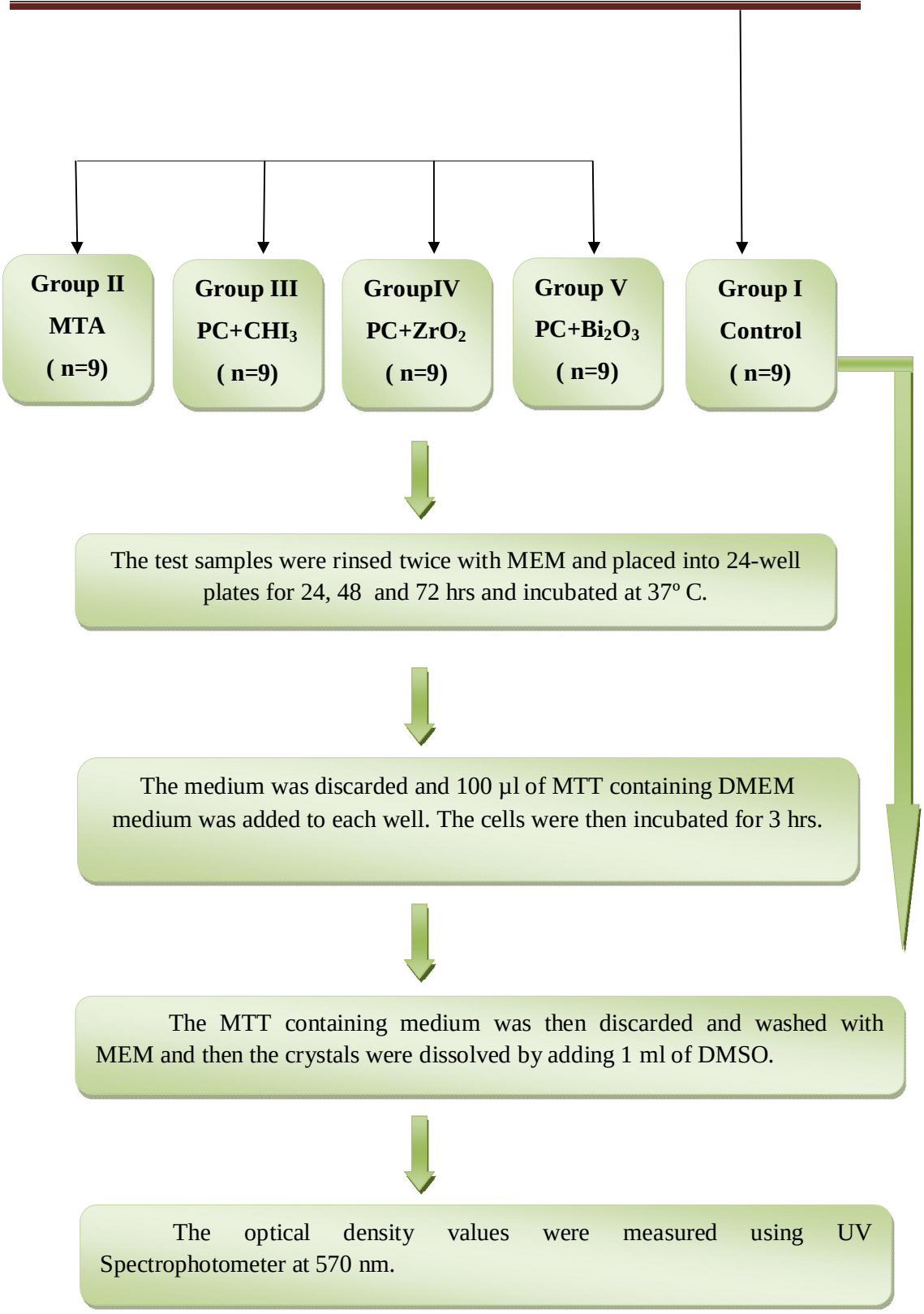
↓

Material were mixed at a concentration of 10 mg/ml and 500µl of this volume was transferred from first to last tube by serial dilution to obtain the 10 desired concentration of the material. (10 mg/ml, 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.2 mg/ml, 0.1 mg/ml, 0.05 mg/ml, 0.02 mg/ml, 0.01 mg/ml, 0.002mg/ml).

↓

Then samples were added into twenty 24 well plates (9 for each groups of 4 groups) and for every different concentration (10 different concentrations for each group of 4 groups)

↓


Group II
MTA
(n=9)
Group III
PC+CHI3
(n=9)
GroupIV
PC+ZrO2
(n=9)
Group V
PC+Bi2O3
(n=9)
Group I
Control
(n=9)
The test samples were rinsed twice with MEM and placed into 24-well plates for 24, 48 and 72 hrs and incubated at 37º C.
The medium was discarded and 100 µl of MTT containing DMEM medium was added to each well. The cells were then incubated for 3 hrs.
The MTT containing medium was then discarded and washed with MEM and then the crystals were dissolved by adding 1 ml of DMSO.
The optical density values were measured using UV Spectrophotometer at 570 nm.

ALP ASSAY

Test materials were added (0.02 mg/ml) & divided into 5 groups of 9 wells each as mentioned for MTT assay

↓

One lakh /ml MC3T3-E1 cells were seeded on test material wells under culture conditions in osteogenic medium

↓

The cells were scraped out from the cells using trypsin/EDTA, washed twice with PBS & resuspended in lysis buffer and centrifuged at 15,000 rpm for 5 min at 4 °C.

↓

Cell lysate was obtained & total protein content was assessed by BCA assay. Then 30 micro gram of protein was used to assess the ALP activity by using p-nitro phenyl phosphate as the substrate.

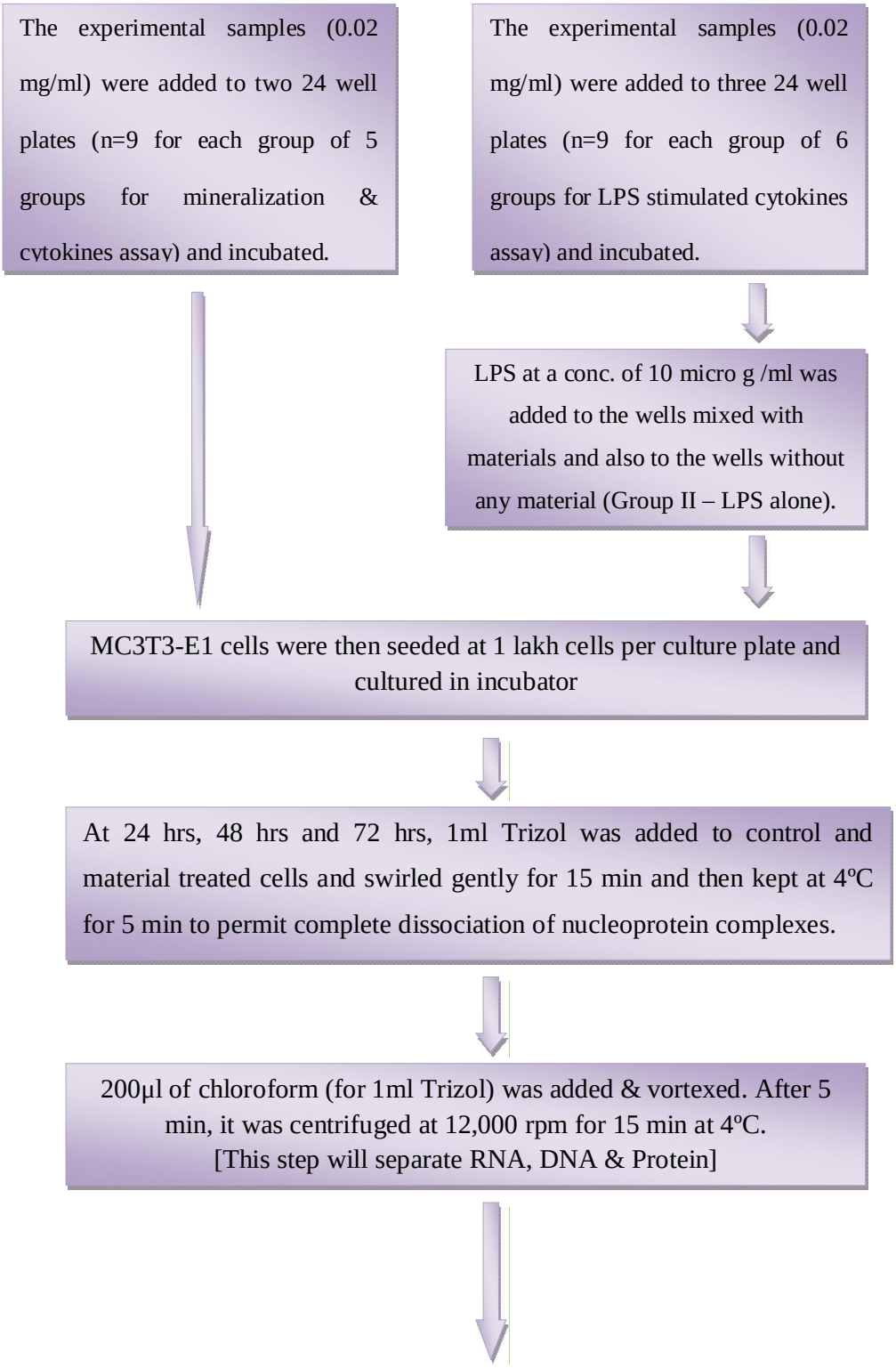
↓

The reaction was stopped by the addition of 1 N NaOH to reaction mixture.

↓

The absorbance at 405 nm (OD value) was measured using UV Spectrophotometer and was expressed as μ moles of ρ-nitrophenol / min / μg protein

Procedural flowchart for qRT-PCR analysis



Then the aqueous phase (contains RNA) was transferred to fresh eppendorf tube & equal amount of Isopropanol was added & centrifuged at 12,000 rpm for 10 min. [This step will precipitate the RNA]

The RNA pellet was washed with 1ml 75% Ethanol to remove any residual acidity and centrifuged at 7500 rpm for 5 min

The pellet was mixed with 25 µl sterile water

Checked at A260 and A280 nm
40µg of RNA when dissolved in 1ml gives 1OD
Therefore the A260 when multiplied by 40 gives the conc. of RNA
A260/A280 gives the purity of RNA (~1.6- 1.8)

2µg of total RNA was Reverse transcribed (cDNA convertion)

The cDNA was used for PCR analysis using gene specific Primer (BSP, OPN, OCN, COL I, TNF a, IL-1 a, IL-6 and GAPDH)

The products were analyzed using Agarose gel electrophoresis for 24, 48 and 72 hours.

ARMAMENTARIUM FOR CYTOTOXICITY AND ALKALINE PHOSPHATASE ACTIVITY

A) CELL CULTURE

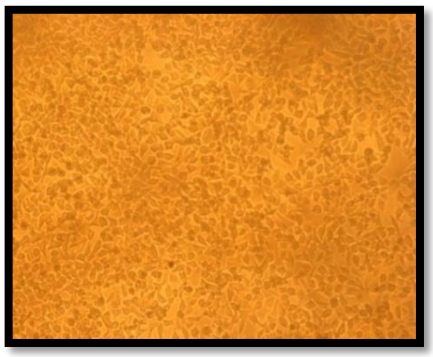


Fig 1& 2: MC3T3-E1 Cell line

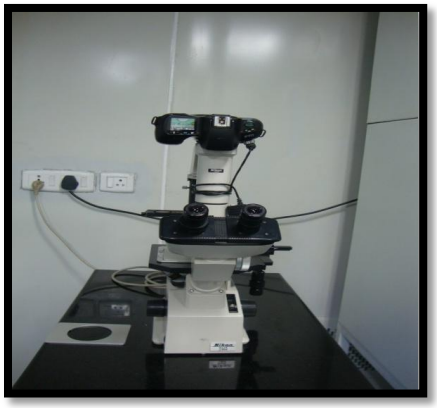


Fig 3: Inverted Phase contrast Microscope

Fig 4: Minimal Essential Media with 10 % FCS

Fig 5: CO_2 Incubator

B) MTT ASSAY

Fig. 6: MTT dye

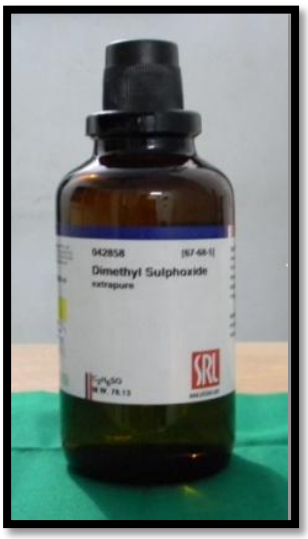


Fig 7: Dimethyl Sulphoxide (DMSO)

C) ALKALINE PHOSPHATASE ASSAY

Fig 8: Aliquots (Supernatants) separated

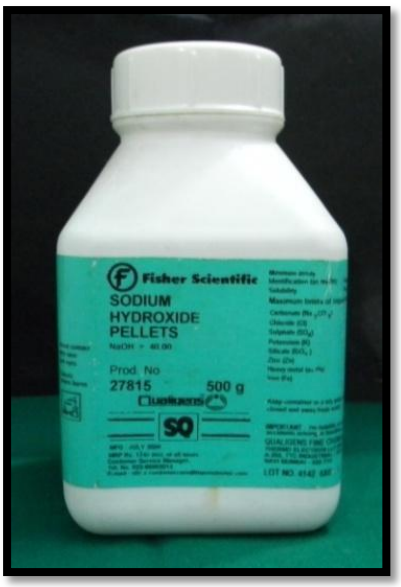


Fig 9 & 10: Reagents for Alkaline Phosphatase Assay

METHODOLOGY FOR CYTOTOXICITY AND ALKALINE PHOSPHATASE ACTIVITY

A) MAINTANENECE OF CELL CULTURE

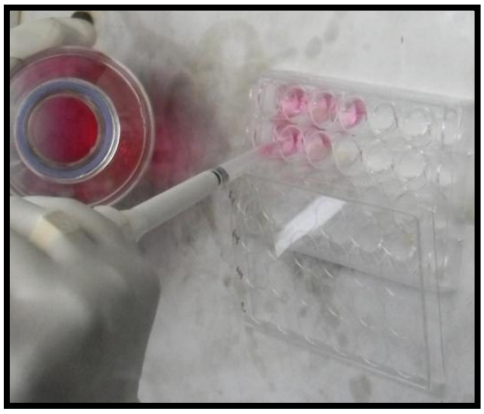


Fig 11: Washing of Samples with Medium

Fig 12: Placement of Cells

B) MTT ASSAY

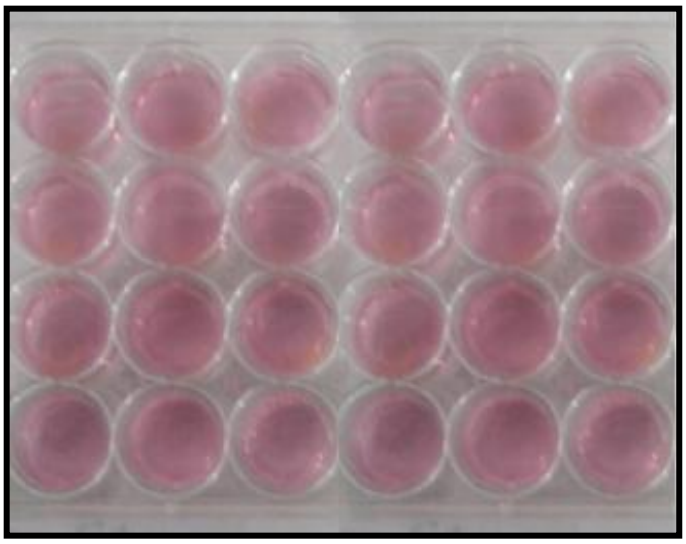


Fig 13: Samples loaded with cells

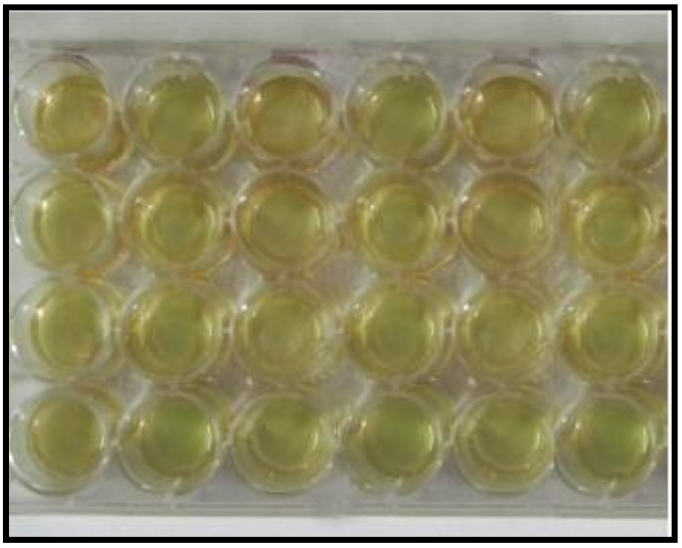


Fig 14: Colour change after MTT reaction

Fig 15: UV Spectrophotometer Showing Reading

C) ALKALINE PHOSPHATASE ASSAY

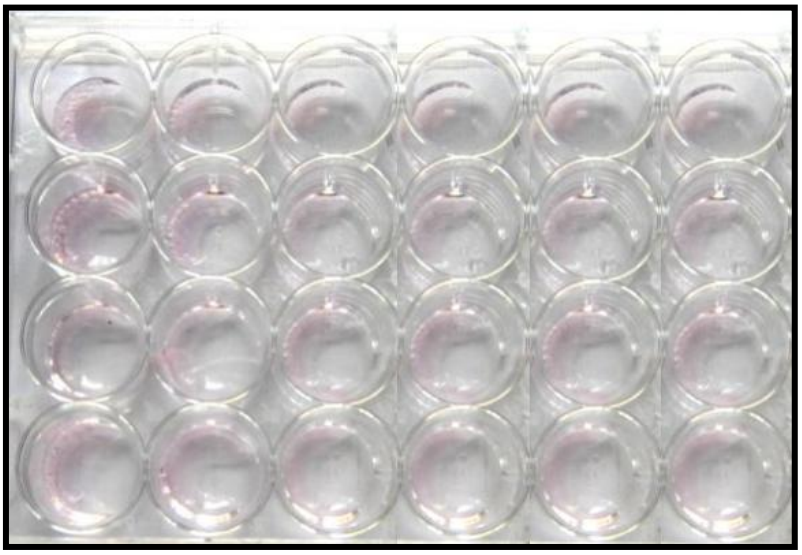


Fig 16: Aliquots placed in 24 well plate

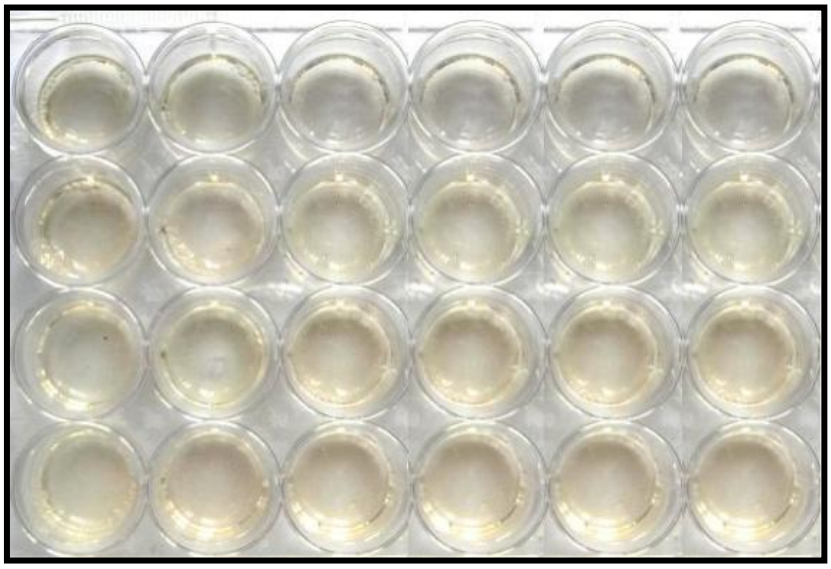


Fig 17: Colour change in Alkaline phosphatase reaction

ARMAMENTARIUM FOR qRT-PCR ANALYSIS

Fig 18 : Gel Documentation system (Bio-Rad)

Fig 19 : Biofuge (Thermo)

Fig. 20 : Thermal cycler (PCR Machine)

Fig. 21: Laboratory Microwave oven

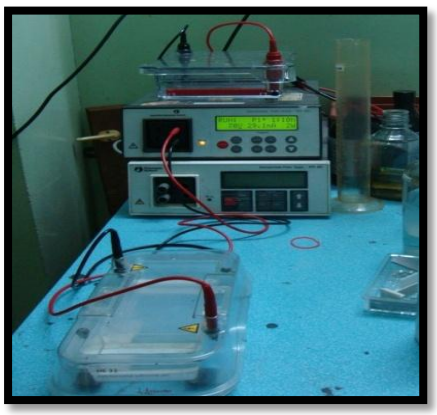


Fig. 22: Electrophoresis unit

Fig. 23: UV visible spectrometer.

METHODOLOGY FOR qRT-PCR ANALYSIS

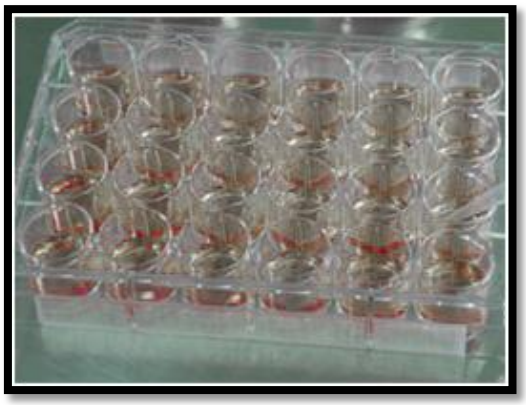


Fig 24 : MC3T3-E1 cells seeded into culture plate

Fig 25 : 1 ml of trizol is added and swirled for 5 min and kept at 4^0C at 24, 48, 72 hrs.

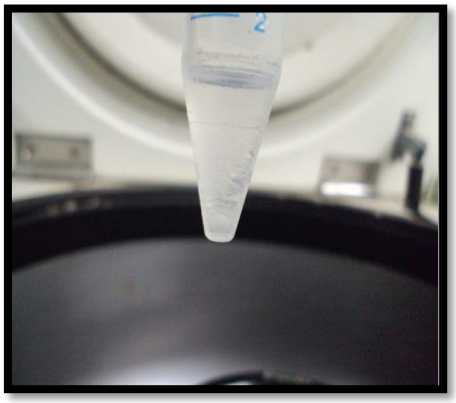


Fig. 26: To the aqueous phase(contains RNA) equal amount of isopropanol is added

Fig 27 : The mixture centrifuged in Biofuge at 12,000 rpm for 10 min to precipitate RNA

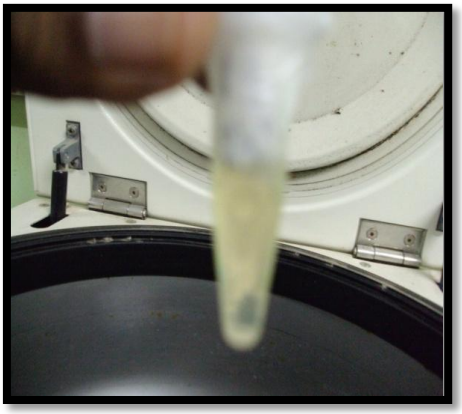


Fig 28: Showing precipitated RNA pellet

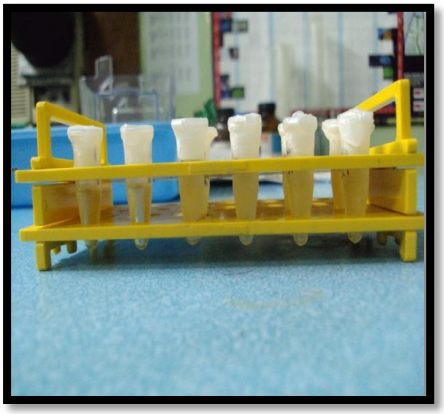


Fig 29: RNA pellet dissolved in RNase and DNase free deionised water and quantified before storing

Fig 30: 2µg of total RNA was Reverse transcribed (cDNA convertion) in thermocycler. Enzyme reverse transcriptase catalyzes the conversion of mRNA into cDNA

Fig 31: Polymerase chain reaction was carried out by setting the annealing temperature and number of cycles

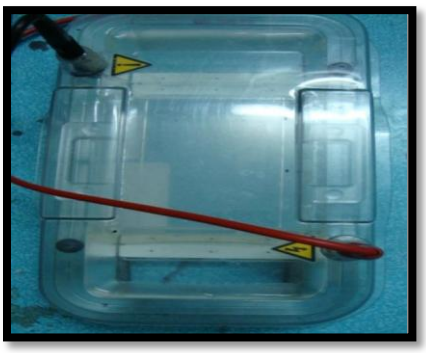


Fig 32: The PCR products were separated using agarose gel electrophoresis

Fig 33: The PCR products visualized using Quantity one software (Bio Rad).

Results

I.Cytotoxicity

Table 1 : Mean ± SD of Cytotoxicity effect of samples on Mouse osteoblastic *cell* line (*MC3T3-E1*)

S.no	Concentration (mg/mL)	24 hrs			
		MTA	**PC + CHI_3**	**PC+ZrO_2**	**PC+Bi_2O_3**
1	10	35.67±0.98	31.44±0.19	37.89±1.98	28.98±1.23
2	2	38.91±0.78	41.32±0.43	40.56±0.78	35.16±0.78
3	1	43.55±0.77	44.16±1.67	43.78±1.76	42.34±1.43
4	0.5	45.89±0.12	46.45±1.98	47.78±0.98	46.90±0.78
5	0.2	48.19±0.18	48.88±0.43	49.78±1.32	49.18±0.98
6	0.1	55.17±0.32	53.21±1.21	51.67±0.54	54.66±1.32
7	0.05	67.89±0.12	63.45±1.98	65.78±0.98	68.90±0.78
8	**0.02**	**72.16±1.67**	**73.61±0.58**	**72.32± 0.67**	**72.22± 0.77**
9	**0.01**	**74.51±0.56**	**75.64±0.91**	**76.12±0.91**	**76.89±1.21**
10	**0.002**	**83.66±0.91**	**85.78±0.65**	**86.78±0.43**	**84.32±1.89**
11	Cell control	86.99± 1.21	86.99± 1.21	86.99±1.21	86.99± 1.21
S.no	**Concentration (mg/mL)**	**48 hrs**			
		MTA	**PC + CHC3**	**PC+2rO_2**	**PC+Bi_2O_3**
1	10	57.89±0.32	49.78±1.23	46.77±0.45	41.89±0.77
2	2	59.08±0.97	54.87±0.76	51.77±0.31	45.67±1.21
3	1	63.78±0.77	64.54±1.01	63.21±0.67	54.32±1.43
4	0.5	64.23±0.12	65.16±0.76	66.46±1.89	55.54±0.81
5	0.2	64.77±0.67	67.88±1.43	68.17±1.43	56.78±1.77
6	0.1	71.56±0.78	71.32±0.54	73.17±1.54	64.32±0.87
7	0.05	74.23±0.12	75.16±0.76	74.89±1.89	74.54±0.81
8	**0.02**	**79.42±.22**	**79.56±0.58**	**79.16± 0.32**	**79.48± 0.72**
9	**0.01**	**81.55±1.49**	**84.13±0.65**	**85.11±1.23**	**89.19±0.78**
10	**0.002**	**86.78±0.88**	**88.98±1.32**	**86.32±0.54**	**90.78±0.76**
11	Cell control	94.42± 0.22	94.42± 0.22	94.42±0.22	94.42± 0.22
S.no	**Concentration (mg/mL)**	**72 hrs**			
		MTA	**PC + CHI_3**	**PC+ZrO_2**	**PC+BI_2O_3**
1	10	81.33±0.54	78.91±1.49	80.78±0.91	83.43±1.22
2	2	84.32±0.32	82.32±1.56	81.56±0.32	84.32±0.91
3	1	84.89±0.67	85.67±1.32	87.65±0.76	86.43±1.43
4	0.5	86.67±0.54	86.32±0.66	88.43±0.87	87.43±0.09
5	0.2	87.43±1.09	89.78±1.7	89.17±0.54	89.76±1.43
6	0.1	89.09±0.98	92.32±1.90	91.67±0.67	91.32±1.99
7	0.05	91.67±0.54	93.32±0.66	93.43±0.87	93.43±0.09
8	**0.02**	**94.51± 0.73**	**94.25± 1.18**	**94.11± 1.36**	**94.9± 0.23**
9	**0.01**	**95.67±0.12**	**98.78±0.62**	**98.17±0.13**	**99.17±0.89**
10	**0.002**	**98.76±0.55**	**99.17±0.89**	**98.78±0.62**	**99.37±0.43**
11	Cell control	78.52+/- 0.78	78.52± 0.78	78.52±0.78	78.52±0.78

Interpretation :

The mean +/- SD of all the OD values obtained from 10 different concentrations of all the experimental groups and cell viability percentage were calculated. The results were as follows,

- The concentrations of all experimental materials including 10, 2, 1, 0.5, 0.2, 0.1 and 0.05 mg/ml showed increased cell survival of osteoblasts from 24 to 72 hrs.
- But 0.02, 0.01 and 0.002 mg/mL concentrations of all experimental materials remarkably increased the cell survival of osteoblasts when compared with the other concentrations of MTA from 24 to 72 hrs.
- Hence the best concentration for all experimental materials was chosen as **0.02 mg/ml** (minimum dilution with best cell survival ability) and was used to compare the cytotoxicity between the groups and also used for alkaline phosphatase assay and qRT-PCR analysis.

Table 2. Mean +/- SD of MTT assay values at a concentration of 0.02 mg/ml of experimental samples at 24, 48 AND 72 HRS

	CONTROL	MTA	PC + CHI3	PC + ZrO2	PC+Bi2O3
24 HRS	86.99+/- 1.21	72.16+/- 1.67	73.61+/- 0.58	72.32+/- 0.67	72.22+/- 0.77
48 HRS	94.42+/- 0.22	99.56+/- 0.58	99.16+/- 0.32	99.48+/- 0.72	99.76+/- 0.50
72 HRS	78.52+/- 0.78	94.51+/- 0.73	94.25+/- 1.18	94.11+/- 1.36	94.9+/- 0.23

Table 3. Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HRS24	Between Groups	502.768	4	125.692	110.892	.000
	Within Groups	11.335	10	1.133		
	Total	514.103	14			
HRS48	Between Groups	58.900	4	14.725	57.635	.000
	Within Groups	2.555	10	.255		
	Total	61.455	14			
HRS72	Between Groups	609.357	4	152.339	170.062	.000
	Within Groups	8.958	10	.896		
	Total	618.315	14			

Table 4. Post Hoc Tests -Multiple Comparisons - Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HRS24	control	MTA	14.8267(*)	.000	11.9658	17.6875
		PC + CHI3	13.3800(*)	.000	10.5191	16.2409
		PC + ZrO2	14.6667(*)	.000	11.8058	17.5275
		PC + Bi2O3	14.7733(*)	.000	11.9125	17.6342
	MTA	control	-14.8267(*)	.000	-17.6875	-11.9658
		PC + CHI3	-1.4467	.494	-4.3075	1.4142
		PC + ZrO2	-.1600	1.000	-3.0209	2.7009
		PC + Bi2O3	-.0533	1.000	-2.9142	2.8075
HRS48	control	MTA	4.8567(*)	.000	3.4984	6.2149
		PC + CHI3	5.2633(*)	.000	3.9051	6.6216
		PC + ZrO2	4.9400(*)	.000	3.5818	6.2982
		PC + Bi2O3	4.6600(*)	.000	3.3018	6.0182
	MTA	control	-4.8567(*)	.000	-6.2149	-3.4984
		PC + CHI3	.4067	.856	-.9516	1.7649
		PC + ZrO2	.0833	1.000	-1.2749	1.4416
		PC + Bi2O3	-.1967	.988	-1.5549	1.1616
HRS72	control	MTA	-15.9833(*)	.000	-18.5266	-13.4400
		PC + CHI3	-15.7267(*)	.000	-18.2700	-13.1834
		PC + ZrO2	-15.5900(*)	.000	-18.1333	-13.0467
		PC + Bi2O3	-16.3800(*)	.000	-18.9233	-13.8367
	MTA	control	15.9833(*)	.000	13.4400	18.5266
		PC + CHI3	.2567	.997	-2.2866	2.8000
		PC + ZrO2	.3933	.985	-2.1500	2.9366
		PC + Bi2O3	-.3967	.984	-2.9400	2.1466

*** The mean difference is significant at the .05 level.**

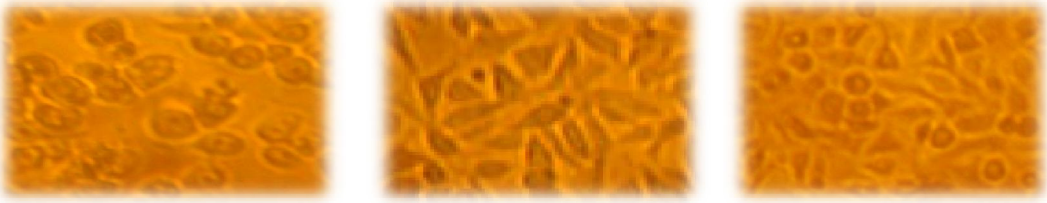
Interpretation:

The mean +/- SD of all OD values at 0.02 mg/ml of experimental sample was obtained and statistical analysis was performed. The results were as follows,

- The cell viability with the presence of all experimental groups was decreased on the first day and second day but increased significantly on the third day compared to the control group.($P<0.01$)
- There was a significant difference between the control group and all experimental groups at all experimental periods. ($p<0.01$).
- There was no statistically significant difference between the experimental groups at all experimental periods.

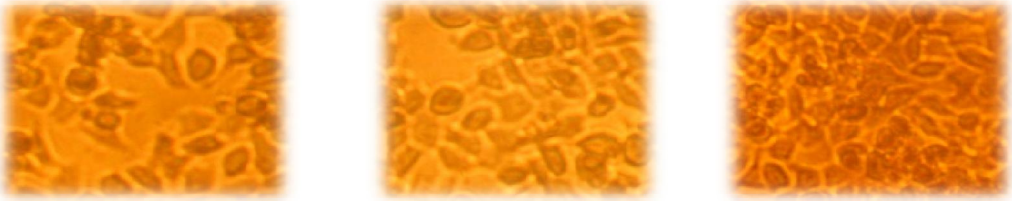
Inverted microscopic view of cell growth at 100 X magnification treated with 0.02 mg/ml of MTA.

24 hrs (0.02mg/mL) **48 hrs (0.02mg/ml)** **72 hrs (0.02mg/ml)**



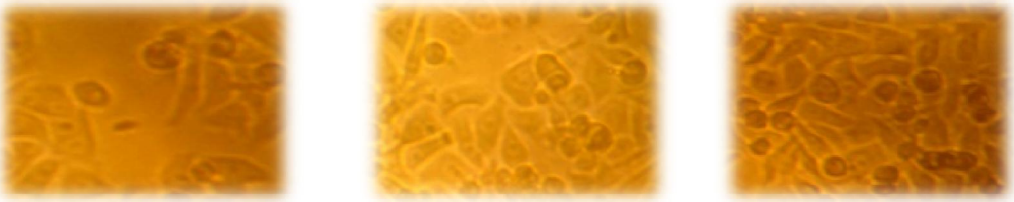
Inverted microscopic view of cell growth at 100 X magnification treated with 0.02 mg/ml of PC + CHI_3.

24 hrs (0.02mg/mL) **48 hrs (0.02mg/ml)** **72 hrs (0.02mg/ml)**



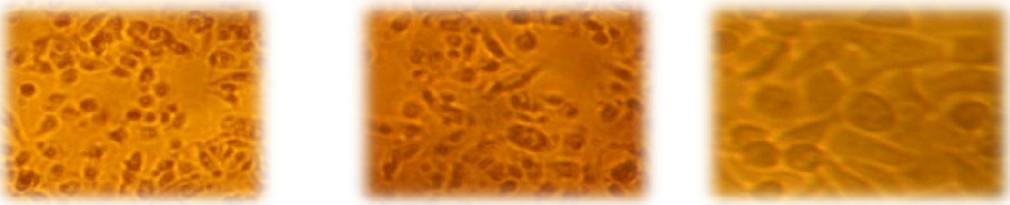
Inverted microscopic view of cell growth at 100 X magnification treated with 0.02 mg/ml of PC + ZrO_2.

24 hrs (0.02mg/mL) **48 hrs (0.02mg/ml)** **72 hrs (0.02mg/ml)**



Inverted microscopic view of cell growth at 100 X magnification treated with 0.02 mg/ml of PC + Bi_2O_3.

24 hrs (0.02mg/mL) **48 hrs (0.02mg/ml)** **72 hrs (0.02mg/ml)**



II.ALKALINE PHOSPHATASE ASSAY

Table 5. Mean +/- SD of ALP values at 3, 7 and 15 days

	CONTROL	MTA	PC + CHI3	PC + ZrO2	PC+Bi2O3
DAY 3	25.37 +/ - 1.02	33.71 +/ - 1.5	35.73 +/ - 0.56	32.76 +/ - 1.52	35.25 +/ - 3.87
DAY 7	55.33 +/ - 1	63.91 +/ - 2.02	63.35 +/ - 1.1	60.51 +/ - 1.71	63.39 +/ - 1.94
DAY 15	60.27 +/ - 1.72	75.81 +/ - 3.38	76.18 +/ - 1.95	73.19 +/ - 2.15	75.35 +/ - 0.81

Table 6. Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
DAY3	Between Groups	210.870	4	52.717	12.572	.001
	Within Groups	41.932	10	4.193		
	Total	252.802	14			
DAY7	Between Groups	154.954	4	38.739	14.838	.000
	Within Groups	26.107	10	2.611		
	Total	181.061	14			
DAY15	Between Groups	546.280	4	136.570	28.998	.000
	Within Groups	47.097	10	4.710		
	Total	593.377	14			

Table 7. Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
DAY3	CONTROL	MTA	-8.3333(*)	.004	-13.8359	-2.8307
		PC + CHI3	-10.3600(*)	.001	-15.8626	-4.8574
		PC +ZrO2	-7.3833(*)	.009	-12.8859	-1.8807
		PC + Bi2O3	-9.8733(*)	.001	-15.3759	-4.3707
	MTA	CONTROL	8.3333(*)	.004	2.8307	13.8359
		PC + CHI3	-2.0267	.745	-7.5293	3.4759
		PC +ZrO2	.9500	.977	-4.5526	6.4526
		PC + Bi2O3	-1.5400	.882	-7.0426	3.9626
DAY7	CONTROL	MTA	-8.5833(*)	.001	-12.9252	-4.2415
		PC + CHI3	-8.0167(*)	.001	-12.3585	-3.6748
		PC +ZrO2	-5.1800(*)	.019	-9.5218	-.8382
		PC + Bi2O3	-8.0600(*)	.001	-12.4018	-3.7182
	MTA	CONTROL	8.5833(*)	.001	4.2415	12.9252
		PC + CHI3	.5667	.992	-3.7752	4.9085
		PC +ZrO2	3.4033	.148	-.9385	7.7452
		PC + Bi2O3	.5233	.994	-3.8185	4.8652

	CONTROL	MTA	-15.5400(*)	.000	-21.3716	-9.7084
		PC + CHI3	-15.9067(*)	.000	-21.7383	-10.0750
		PC +ZrO2	-12.9233(*)	.000	-18.7550	-7.0917
DAY15		PC + Bi2O3	-15.0833(*)	.000	-20.9150	-9.2517
	MTA	CONTROL	15.5400(*)	.000	9.7084	21.3716
		PC + CHI3	-.3667	1.000	-6.1983	5.4650
		PC +ZrO2	2.6167	.598	-3.2150	8.4483
		PC + Bi2O3	.4567	.999	-5.3750	6.2883

*** The mean difference is significant at the .05 level.**

Interpretation:

The mean +/- SD of all OD values for ALP assay at 0.02 mg/ml concentration of the experimental sample was obtained and stastical analysis was performed. The results were as follows,

- There was a significant difference between the control groups and all the experimental groups ($p<0.01$) at day 3, day 7 and day 15.
- There was no statistically significant difference between the experimental groups at day 3, day 7 and day 15.
- The OD values in ALP assay was significantly increased from day 3 to day 15 ($p<0.01$) for all the experimental groups.

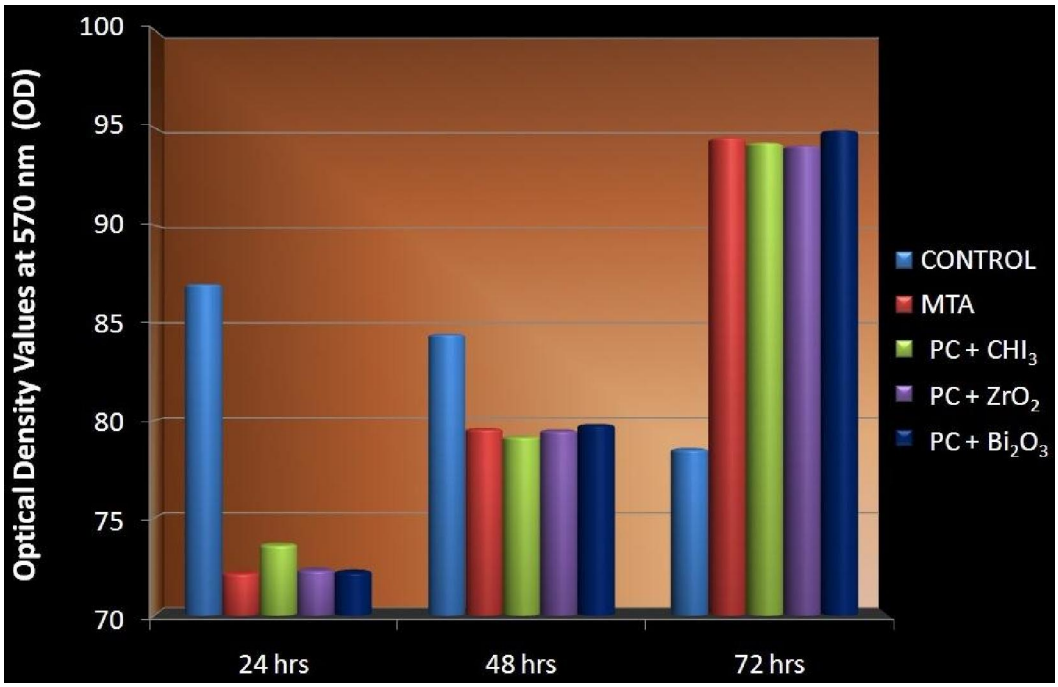


Chart-1 : Cytotoxicity of Mouse osteoblast cell lines (MC3T3-E1) at 24, 48 and 72 hrs

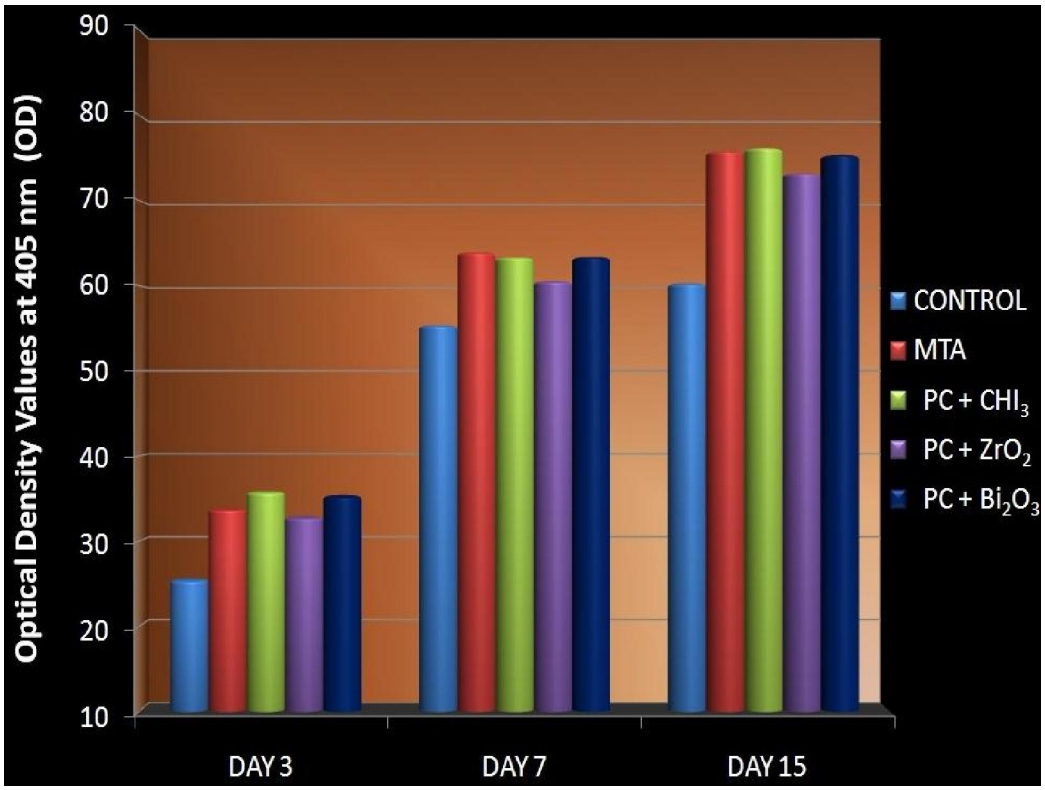


Chart-2 : ALP assay of Mouse osteoblast cell lines (MC3T3-E1) at 3, 7 and 15 days

III.qRT-PCR ANALYSIS

a) Mineralization associated proteins

i) BSP

Table 8 : Mean +/- SD of OD values expressing mRNA of BSP

	CONTROL	MTA	PC + CHI 3	PC + ZrO2	PC + Bi2O3
24 HRS	87.03 + / - 0.2	33.1 + / - 1.7	34.38 + / - 2.3	33.81 + / - 0.68	33.89 + / - 1.2
48 HRS	86.46 + / - 1.3	60.8 + / - 2.1	60.73 + / - 1.3	61.32 + / - 1.07	61.66 + / - 1.6
72 HRS	85.79 + / - 1.8	83.8 + / - 2.1	83.7 + / - 1.35	84.32 + / - 1.07	84.66 + / - 1.6

Table 9 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HRS24	Between Groups	6804.620	4	1701.155	790.544	.000
	Within Groups	21.519	10	2.152		
	Total	6826.138	14			
HRS48	Between Groups	1539.231	4	384.808	166.992	.000
	Within Groups	23.043	10	2.304		
	Total	1562.274	14			
HRS72	Between Groups	8.092	4	2.023	.761	.574
	Within Groups	26.583	10	2.658		
	Total	34.676	14			

Table 10 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HRS24	control	MTA	53.9267(*)	.000	49.9848	57.8685
		PC + CHI3	52.6567(*)	.000	48.7148	56.5985
		PC + ZrO2	53.2233(*)	.000	49.2815	57.1652
		PC + Bi2O3	53.1433(*)	.000	49.2015	57.0852
	MTA	control	-53.9267(*)	.000	-57.8685	-49.9848
		PC + CHI3	-1.2700	.822	-5.2119	2.6719
		PC + ZrO2	-.7033	.974	-4.6452	3.2385
		PC + Bi2O3	-.7833	.962	-4.7252	3.1585
HRS48	control	MTA	25.5767(*)	.000	21.4975	29.6558
		PC + CHI3	25.7267(*)	.000	21.6475	29.8058
		PC + ZrO2	25.1433(*)	.000	21.0642	29.2225
		PC + Bi2O3	24.8000(*)	.000	20.7209	28.8791
	MTA	control	-25.5767(*)	.000	-29.6558	-21.4975
		PC + CHI3	.1500	1.000	-3.9291	4.2291
		PC + ZrO2	-.4333	.996	-4.5125	3.6458
		PC + Bi2O3	-.7767	.967	-4.8558	3.3025
		PC + ZrO2	.3433	.998	-3.7358	4.4225

HRS72	control	MTA	1.9100	.621	-2.4713	6.2913
		PC + CHI3	2.0600	.558	-2.3213	6.4413
		PC + ZrO2	1.4767	.798	-2.9046	5.8579
		PC + Bi2O3	1.1333	.908	-3.2479	5.5146

ii) OPN

Table 11: Mean +/- SD of OD values expressing mRNA of OPN values

	CONTROL	MTA	PC + CHI 3	PC + ZrO2	PC + Bi2O3
24 HRS	87.03 + / - 0.22	55.39 + / - 0.96	51.51 + / - 0.57	53.87 + / - 2.5	54.17 + / - 2.3
48 HRS	86.46 + / - 1.31	64.88 + / - 2.01	64.73 + / - 1.35	65.32 + / - 1.1	65.66 + / - 1.6
72 HRS	85.79 + / - 1.87	89.88 + / - 2.01	89.73 + / - 1.35	90.32 + / - 1.2	90.66 + / - 1.6

Table 12 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HRS24	Between Groups	2684.428	4	671.107	248.834	.000
	Within Groups	26.970	10	2.697		
	Total	2711.398	14			
HRS48	Between Groups	1091.647	4	272.912	118.433	.000
	Within Groups	23.043	10	2.304		
	Total	1114.690	14			
HRS72	Between Groups	47.116	4	11.779	4.431	.026
	Within Groups	26.583	10	2.658		
	Total	73.700	14			

Table 13 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HRS24	control	MTA	31.6467(*)	.000	27.2337	36.0597
		PC + CHI3	35.5200(*)	.000	31.1070	39.9330
		PC + ZrO2	33.1600(*)	.000	28.7470	37.5730
		PC + Bi2O3	32.8600(*)	.000	28.4470	37.2730
	MTA	control	-31.6467(*)	.000	-36.0597	-27.2337
		PC + CHI3	3.8733	.093	-.5397	8.2863
		PC + ZrO2	1.5133	.789	-2.8997	5.9263
		PC + Bi2O3	1.2133	.889	-3.1997	5.6263
HRS48	control	MTA	21.5767(*)	.000	17.4975	25.6558
		PC + CHI3	21.7267(*)	.000	17.6475	25.8058
		PC + ZrO2	21.1433(*)	.000	17.0642	25.2225
		PC + Bi2O3	20.8000(*)	.000	16.7209	24.8791
	MTA	control	-21.5767(*)	.000	-25.6558	-17.4975
		PC + CHI3	.1500	1.000	-3.9291	4.2291
		PC + ZrO2	-.4333	.996	-4.5125	3.6458
		PC + Bi2O3	-.7767	.967	-4.8558	3.3025

HRS72	control	MTA	-4.0900(*)	.040	-8.4713	.2913
		PC + CHI3	-3.9400(*)	.043	-8.3213	.4413
		PC + ZrO2	-4.5233(*)	.042	-8.9046	-.1421
		PC + Bi2O3	-4.8667(*)	.028	-9.2479	-.4854

iii) OCN

Table 14 : Mean +/- SD of of OD values expressing mRNA of OCN values

	CONTROL	MTA	PC + CHI 3	PC + ZrO2	Pc + Bi2O3
24 HRS	87.03 +/- 0.22	49.57 +/- 2.13	48.82+/- 4.08	51.80 +/- 1.23	51.30 +/- 2.063
48 HRS	86.46 +/- 1.31	62.88 +/- 2.01	62.73+/- 1.35	63.32 +/- 1.07	63.66 +/- 1.66
72 HRS	85.79 +/- 1.87	84.88 +/- 2.01	84.73/- 1.35	85.32 +/- 1.07	85.66 +/- 1.66

Table 15 : Oneway ANOVA

		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
HRS24	Between Groups	3243.334	4	810.834	149.600	.000
	Within Groups	54.200	10	5.420		
	Total	3297.534	14			
HRS48	Between Groups	1305.839	4	326.460	141.671	.000
	Within Groups	23.043	10	2.304		
	Total	1328.882	14			
HRS72	Between Groups	10.015	4	2.504	1.292	.337
	Within Groups	19.383	10	1.938		
	Total	29.398	14			

Table 16 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	**(I) GROUPS**	**(J) GROUPS**	**Mean Difference (I-J)**	**Sig.**	**95% Confidence Interval**	
					Lower Bound	**Upper Bound**
HRS24	control	MTA	37.4600(*)	.000	31.2040	43.7160
		PC + CHI3	38.2133(*)	.000	31.9574	44.4693
		PC + ZrO2	35.2333(*)	.000	28.9774	41.4893
		PC + Bi2O3	35.7333(*)	.000	29.4774	41.9893
	MTA	control	-37.4600(*)	.000	-43.7160	-31.2040
		PC + CHI3	.7533	.994	-5.5026	7.0093
		PC + ZrO2	-2.2267	.767	-8.4826	4.0293
		PC + Bi2O3	-1.7267	.887	-7.9826	4.5293
HRS48	control	MTA	23.5767(*)	.000	19.4975	27.6558
		PC + CHI3	23.7267(*)	.000	19.6475	27.8058
		PC + ZrO2	23.1433(*)	.000	19.0642	27.2225
		PC + Bi2O3	22.8000(*)	.000	18.7209	26.8791

	MTA	control	-23.5767(*)	.000	-27.6558	-19.4975
		PC + CHI3	.1500	1.000	-3.9291	4.2291
		PC + ZrO2	-.4333	.996	-4.5125	3.6458
		PC + Bi2O3	-.7767	.967	-4.8558	3.3025
HRS72	control	MTA	2.2433	.343	-1.4978	5.9845
		PC + CHI3	1.0600	.878	-2.6812	4.8012
		PC + ZrO2	.4767	.992	-3.2645	4.2178
		PC + Bi2O3	.1333	1.000	-3.6078	3.8745

iv) COL I

Table 17 : Mean +/- SD of OD values expressing mRNA of COL I values

	CONTROL	MTA	PC + CHI 3	PC + ZrO2	Pc + Bi2O3
24 HRS	87.03 + / - 0.22	23.05 + / - 1.34	22.59+ / - 0.83	23.84 + / - 0.11	23.45 + / - 1.31
48 HRS	86.46 + / - 1.31	42.88 + / - 2.01	42.73+ / - 1.35	43.32 + / - 1.07	43.66 + / - 1.66
72 HRS	85.79 + / - 1.87	82.88+ / - 2.01	82.73+ / - 1.35	83.65 + / - 1.61	83.66 + / - 1.66

Table 18 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
HRS24	Between Groups	9771.639	4	2442.910	2850.714	.000
	Within Groups	8.569	10	.857		
	Total	9780.208	14			
HRS48	Between Groups	4503.759	4	1125.940	488.616	.000
	Within Groups	23.043	10	2.304		
	Total	4526.802	14			
HRS72	Between Groups	17.934	4	4.483	1.519	.269
	Within Groups	29.510	10	2.951		
	Total	47.444	14			

Table 19 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HRS24	control	MTA	63.9867(*)	.000	61.4991	66.4742
		PC + CHI3	64.4400(*)	.000	61.9525	66.9275
		PC + ZrO2	63.1933(*)	.000	60.7058	65.6809
		PC + Bi2O3	63.5800(*)	.000	61.0925	66.0675
	MTA	control	-63.9867(*)	.000	-66.4742	-61.4991
		PC + CHI3	.4533	.972	-2.0342	2.9409
		PC + ZrO2	-.7933	.827	-3.2809	1.6942
		PC + Bi2O3	-.4067	.981	-2.8942	2.0809
HRS48	control	MTA	43.5767(*)	.000	39.4975	47.6558
		PC + CHI3	43.7267(*)	.000	39.6475	47.8058

		PC + ZrO2	43.1433(*)	.000	39.0642	47.2225
		PC + Bi2O3	42.8000(*)	.000	38.7209	46.8791
	MTA	control	-43.5767(*)	.000	-47.6558	-39.4975
		PC + CHI3	.1500	1.000	-3.9291	4.2291
		PC + ZrO2	-.4333	.996	-4.5125	3.6458
		PC + Bi2O3	-.7767	.967	-4.8558	3.3025
		PC + ZrO2	.3433	.998	-3.7358	4.4225
HRS72	control	MTA	2.9100	.301	-1.7061	7.5261
		PC + CHI3	3.0600	.261	-1.5561	7.6761
		PC + ZrO2	2.1433	.569	-2.4728	6.7595
		PC + Bi2O3	2.1333	.573	-2.4828	6.7495

Interpretation of Results obtained for Mineralization proteins:

The mean +/- SD of all OD values relative to GADPHwas obtained and stastical analysis was performed. The results were as follows,

i) BSP

- The BSP expression level declined in both MTA and other experimental groups compared with the control group on the first and second day. ($p<0.01$)
- However, gene expression recovered to the control level in both MTA and other experimental groups on the third day. .($p=0.574$)
- The gene expression between the experimental groups was statistically insignificant.

ii) OPN

- The OPN expression levels in the MTA and other experimental groups decreased significantly compared with the control group on the first day and second day. ($p<0.01$)

- By the third day, OPN expression was significantly enhanced in both MTA and other experimental groups compared with the control groups.($p=0.026$)
- The gene expression between the experimental groups was statistically insignificant.

iii) OCN

- The OCN expression levels declined in both MTA and other experimental groups compared with the control group on the first and second day. ($p<0.01$)
- However, gene expression recovered to the control level in both MTA and other experimental groups on the third day. ($p=0.337$)
- The gene expression between the experimental groups was statistically insignificant.

iv) **COL I**

- The COL I expression levels declined in both MTA and other experimental groups compared with the control group on the first and second day. . ($p<0.01$)
- However, gene expression recovered to the control level in both MTA and other experimental groups on the third day. ($p=0.269$).
- The gene expression between the experimental groups was statistically insignificant.

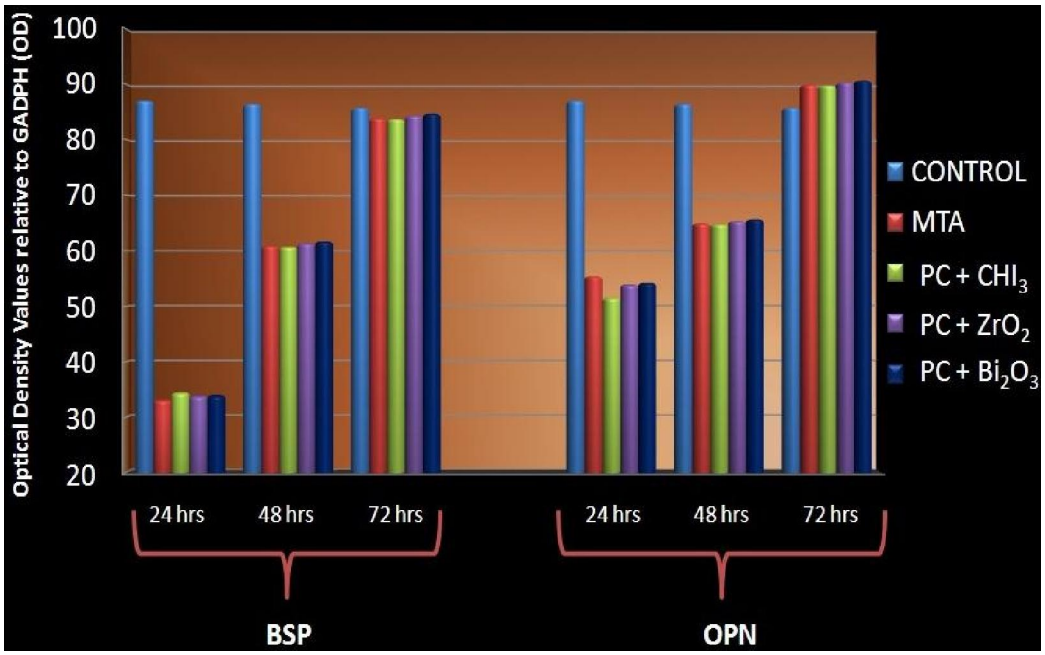


Chart-3 : mRNA expression of Mineralization associated proteins BSP & OPN analyzed by qRT-PCR at 24, 48 and 72 hrs.

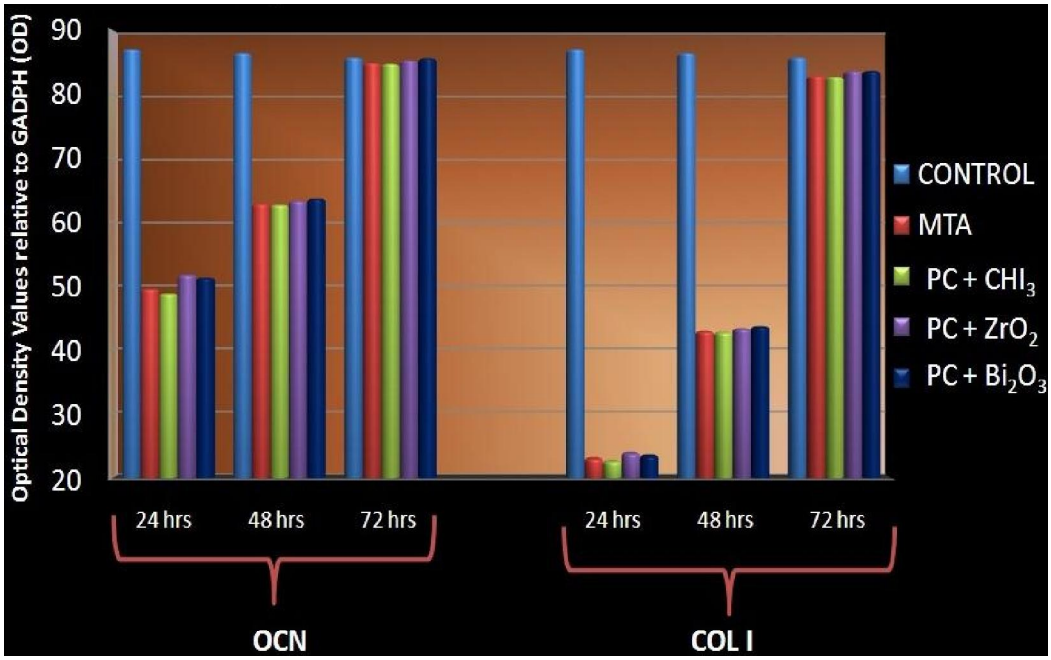
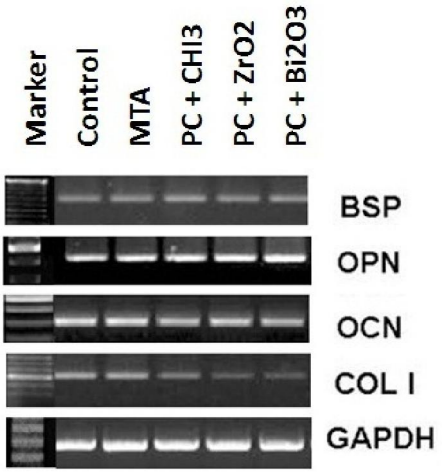
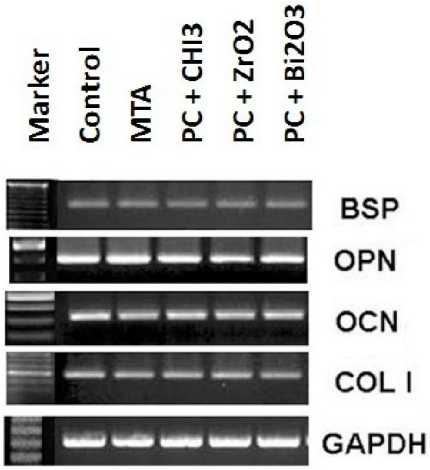


Chart-4 : mRNA expression of Mineralization associated proteins OCN & COL I analyzed by qRT-PCR at 24, 48 and 72 hrs.

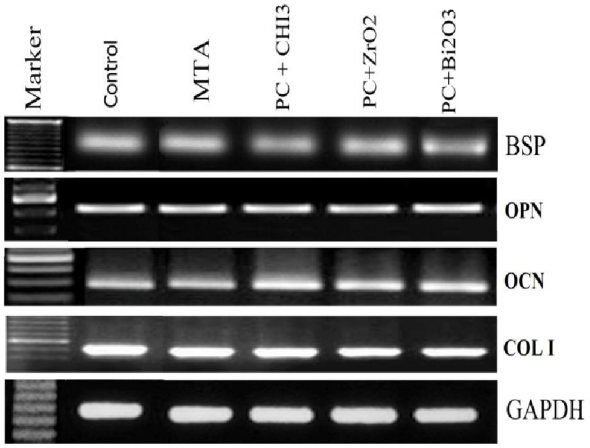
mRNA expression of Mineralization associated proteins by qRT-PCR analysis at 24, 48 and 72 hrs. GAPDH was used as housekeeping gene.



24 hrs



48 hrs



72 hrs

a) Cytokines involved in Mineralization

- **24 hrs**

Table 20 : Mean +/- SD of OD values expressing mRNA of cytokines at 24 HRS

	CONTROL	MTA	PC + CHI3	PC + ZrO2	PC+Bi2O3
TNF α	43.69 +/- 1.78	34.6+/- 1.76	34.89 +/- 0.7	34.3 +/- 0.7	34.4+/- 0.66
IL-6	54.48 +/- 1.28	47.03 +/- 0.22	46.93 +/- 0.9	47.5+/- 1.2	46.87+/- 0.6
IL-1 α	18.66 +/- 0.3	3.34+/- 0.19	3.59+/- 0.41	3.51+/- 0.3	3.55+/- 0.37

Table 21 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF α	Between Groups	200.311	4	50.078	31.655	.010
	Within Groups	15.820	10	1.582		
	Total	216.131	14			
IL-6	Between Groups	132.180	4	33.045	35.502	.010
	Within Groups	9.308	10	.931		
	Total	141.487	14			
IL-1 α	Between Groups	552.060	4	138.015	1217.850	.000
	Within Groups	1.133	10	.113		
	Total	553.193	14			

Table 22 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
TNF α					Lower Bound	Upper Bound
	CONTROL	MTA	9.0967(*)	.000	5.7168	12.4765
		PC + CHI3	8.8067(*)	.000	5.4268	12.1865
		PC + ZrO2	9.3633(*)	.000	5.9835	12.7432
		PC + Bi2O3	9.2300(*)	.000	5.8502	12.6098
	MTA	CONTROL	-9.0967(*)	.000	-12.4765	-5.7168
		PC + CHI3	-.2900	.998	-3.6698	3.0898
		PC + ZrO2	.2667	.999	-3.1132	3.6465
		PC + Bi2O3	.1333	1.000	-3.2465	3.5132
IL-6	CONTROL	MTA	7.4500(*)	.000	4.8575	10.0425
		PC + CHI3	7.5567(*)	.000	4.9642	10.1492
		PC + ZrO2	6.9867(*)	.000	4.3942	9.5792
		PC + Bi2O3	7.6100(*)	.000	5.0175	10.2025
	MTA	CONTROL	-7.4500(*)	.000	-10.0425	-4.8575
		PC + CHI3	.1067	1.000	-2.4858	2.6992
		PC + ZrO2	-.4633	.974	-3.0558	2.1292

		PC + Bi2O3	.1600	1.000	-2.4325	2.7525
IL-1 α	CONTROL	MTA	15.3267(*)	.000	14.4221	16.2313
		PC + CHI3	15.0700(*)	.000	14.1654	15.9746
		PC + ZrO2	15.1500(*)	.000	14.2454	16.0546
		PC + Bi2O3	15.1133(*)	.000	14.2087	16.0179
	MTA	CONTROL	-15.3267(*)	.000	-16.2313	-14.4221
		PC + CHI3	-.2567	.877	-1.1613	.6479
		PC + ZrO2	-.1767	.964	-1.0813	.7279
		PC + Bi2O3	-.2133	.932	-1.1179	.6913

- **48 hrs**

Table 23 : Mean +/- SD of OD values expressing mRNA of cytokines at 48 HRS

	CONTROL	MTA	PC + CHI3	PC + ZrO2	PC+Bi2O3
TNF α	76.21 +/- 2.44	54.6 +/- 1.7	54.89 +/- 0.7	54.33 +/- 0.7	54.46 +/- 0.6
IL-6	65.05 +/- 1.60	67.03 +/- 0.2	66.93 +/- 0.9	67.5 +/- 1.26	66.87 +/- 0.6
IL-1 α	9.50 +/- 0.25	2.34 +/- 0.1	2.59 +/- 0.41	2.51 +/- 0.34	2.55 +/- 0.37

Table 24 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF α	Between Groups	1124.838	4	281.209	131.288	.010
	Within Groups	21.419	10	2.142		
	Total	1146.257	14			
IL-6	Between Groups	10.640	4	2.660	2.380	.121
	Within Groups	11.179	10	1.118		
	Total	21.819	14			
IL-1 α	Between Groups	2.538	4	.635	5.887	.001
	Within Groups	1.078	10	.108		
	Total	3.616	14			

Table 25 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TNF α	CONTROL	MTA	21.6167(*)	.000	17.6839	25.5494
		PC + CHI3	21.3267(*)	.000	17.3939	25.2594
		PC + ZrO2	21.8833(*)	.000	17.9506	25.8161
		PC + Bi2O3	21.7500(*)	.000	17.8173	25.6827
	MTA	CONTROL	-21.6167(*)	.000	-25.5494	-17.6839
		PC + CHI3	-.2900	.999	-4.2227	3.6427

		PC + ZrO2	.2667	.999	-3.6661	4.1994
		PC + Bi2O3	.1333	1.000	-3.7994	4.0661
IL-6	CONTROL	MTA	-1.9833	.222	-4.8245	.8578
		PC + CHI3	-1.8767	.264	-4.7178	.9645
		PC + ZrO2	-2.4467	.101	-5.2878	.3945
		PC + Bi2O3	-1.8233	.286	-4.6645	1.0178
IL-1 α	CONTROL	MTA	1.1667(*)	.010	.2844	2.0489
		PC + CHI3	.9100(*)	.043	.0278	1.7922
		PC + ZrO2	.9900(*)	.027	.1078	1.8722
		PC + Bi2O3	.9533(*)	.033	.0711	1.8356
	MTA	CONTROL	-1.1667(*)	.010	-2.0489	-.2844
		PC + CHI3	-.2567	.868	-1.1389	.6256
		PC + ZrO2	-.1767	.961	-1.0589	.7056
		PC + Bi2O3	-.2133	.926	-1.0956	.6689

- **72 hrs**

Table 26 : Mean +/- SD of OD values expressing mRNA of cytokines at 72 HRS

	CONTROL	MTA	PC + CHI3	PC + ZrO2	PC+Bi2O3
TNF α	86.28 +/- 1.77	74.6 +/- 1.7	74.89+/- 0.7	74.33+/- 0.7	74.46+/- 0.66
IL-6	74.39+/- 1.22	87.03+/- 0.22	86.93+/- 0.9	87.5+/- 1.26	86.87+/- 0.61
IL-1 α	6.29+/- 0.15	1.34+/- 0.19	1.59+/- 0.41	1.51+/- 0.34	1.55+/- 0.37

Table 27 : Oneway ANOVA

		Sum of Squares	Df	Mean Square	F	Sig.
TNF α	Between Groups	329.842	4	82.460	52.188	.010
	Within Groups	15.801	10	1.580		
	Total	345.643	14			
IL-6	Between Groups	387.365	4	96.841	107.709	.010
	Within Groups	8.991	10	.899		
	Total	396.356	14			
IL-1 α	Between Groups	55.218	4	13.805	138.619	.000
	Within Groups	.996	10	.100		
	Total	56.214	14			

Table 28 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TNF α	CONTROL	MTA	11.6867(*)	.000	8.3089	15.0644
		PC + CHI3	11.3967(*)	.000	8.0189	14.7744

		PC + ZrO2	11.9533(*)	.000	8.5756	15.3311
		PC + Bi2O3	11.8200(*)	.000	8.4422	15.1978
	MTA	CONTROL	-11.6867(*)	.000	-15.0644	-8.3089
		PC + CHI3	-.2900	.998	-3.6678	3.0878
		PC + ZrO2	.2667	.999	-3.1111	3.6444
		PC + Bi2O3	.1333	1.000	-3.2444	3.5111
IL-6	CONTROL	MTA	-12.6433(*)	.000	-15.1913	-10.0953
		PC + CHI3	-12.5367(*)	.000	-15.0847	-9.9887
		PC + ZrO2	-13.1067(*)	.000	-15.6547	-10.5587
		PC + Bi2O3	-12.4833(*)	.000	-15.0313	-9.9353
	MTA	CONTROL	12.6433(*)	.000	10.0953	15.1913
		PC + CHI3	.1067	1.000	-2.4413	2.6547
		PC + ZrO2	-.4633	.972	-3.0113	2.0847
		PC + Bi2O3	.1600	1.000	-2.3880	2.7080
IL-1 α	CONTROL	MTA	4.9533(*)	.000	4.1053	5.8013
		PC + CHI3	4.6967(*)	.000	3.8487	5.5447
		PC + ZrO2	4.7767(*)	.000	3.9287	5.6247
		PC + Bi2O3	4.7400(*)	.000	3.8920	5.5880
	MTA	CONTROL	-4.9533(*)	.000	-5.8013	-4.1053
		PC + CHI3	-.2567	.851	-1.1047	.5913
		PC + ZrO2	-.1767	.955	-1.0247	.6713
		PC + Bi2O3	-.2133	.916	-1.0613	.6347

Interpretation

The mean +/- SD of all OD values relative to GADPH was obtained and stastical analysis was performed. The results were as follows,

At 24 hrs,

- The qRT-PCR analysis detected moderate to high levels of IL-6 and TNF alpha on all the groups but significantly less than the control group ($p<0.01$).
- IL-1alpha was more detected in control group and its reduction in the presence of experimental groups was highly significant ($p=0$).
- The expression of all cytokines between the experimental groups was statistically insignificant.

At 48 hrs,

- The qRT-PCR analysis detected moderate to high levels of TNF alpha on all the groups but significantly less than the control group ($p<0.01$).

- IL-1alpha was more detected in control group and its reduction in the presence of experimental groups was highly significant (p=0).
- At 48 hrs, the level of IL-6 in all the experimental groups was insignificant with the control group. (p=0.121).
- The expression of all cytokines between the experimental groups was statistically insignificant.

At 72 hrs,

- The qRT-PCR analysis detected moderate to high levels of TNF alpha on all the groups but significantly less than the control group (p<0.01).
- IL-1alpha was more detected in control group and its reduction in the presence of experimental groups was highly significant (p=0).
- At 72 hrs, the level of IL-6 was increased significantly in all the experimental groups than the control group. (p=0.01).
- The expression of all cytokines between the experimental groups was statistically insignificant.

b) Cytokines involved in Mineralization (LPS stimulated)

- **24 hrs**

Table 29 : Mean +/- SD of OD values expressing mRNA of cytokines at 24 HRS

	CONTROL	LPS alone	LPS + MTA	LPS + PC + CHI3	LPS + PC + ZrO2	LPS + PC+Bi2O3
TNF α	43.69± 1.78	18.5± 0.53	33.81±0.68	33.89 ± 1.25	34.52± 0.53	35.57 ± 1.32
IL-6	54.48± 1.28	23.88± 0.62	34.40± 1.17	34.38±1.25	35.12± 0.66	34.74± 0.41
IL-1 α	18.66± 0.30	42.87± 1.64	8.7± 0.24	8.74 ± 0.07	8.77± 0.12	8.50± 0.54

Table 30 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF α	Between Groups	1003.168	5	200.634	159.94	.000
	Within Groups	15.053	12	1.254		

	Total	1018.221	17			
IL-6	Between Groups	1487.453	5	297.491	318.90	.000
	Within Groups	11.194	12	.933		
	Total	1498.647	17			
IL-1 α	Between Groups	2816.147	5	563.229	1169.9	.000
	Within Groups	5.777	12	.481		
	Total	2821.924	17			

Table 31 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TNF α	CONTROL	LPS ALONE	25.1967(*)	.000	22.1250	28.2683
		LPS + MTA	9.8833(*)	.000	6.8117	12.9550
		LPS + PC + CHI3	9.8033(*)	.000	6.7317	12.8750
		LPS+ PC + ZrO2	9.1733(*)	.000	6.1017	12.2450
		LPS + PC + Bi2O3	8.1267(*)	.000	5.0550	11.1983
	LPS alone	CONTROL	-25.1967(*)	.000	-28.2683	-22.1250
		LPS + MTA	-15.3133(*)	.000	-18.3850	-12.2417
		LPS + PC + CHI3	-15.3933(*)	.000	-18.4650	-12.3217
		LPS+ PC + ZrO2	-16.0233(*)	.000	-19.0950	-12.9517
		LPS + PC + Bi2O3	-17.0700(*)	.000	-20.1417	-13.9983
	LPS + MTA	CONTROL	-9.8833(*)	.000	-12.9550	-6.8117
		LPS ALONE	15.3133(*)	.000	12.2417	18.3850
		LPS + PC + CHI3	-.0800	1.000	-3.1517	2.9917
		LPS+ PC + ZrO2	-.7100	.967	-3.7817	2.3617
		LPS + PC + Bi2O3	-1.7567	.435	-4.8283	1.3150
IL-6	CONTROL	LPS ALONE	30.6000(*)	.000	27.9511	33.2489
		LPS + MTA	20.0800(*)	.000	17.4311	22.7289
		LPS + PC + CHI3	20.1000(*)	.000	17.4511	22.7489
		LPS+ PC + ZrO2	19.3667(*)	.000	16.7178	22.0155
		LPS + PC + Bi2O3	19.7467(*)	.000	17.0978	22.3955
	LPS alone	CONTROL	-30.6000(*)	.000	-33.2489	-27.9511
		LPS + MTA	-10.5200(*)	.000	-13.1689	-7.8711
		LPS + PC + CHI3	-10.5000(*)	.000	-13.1489	-7.8511
		LPS+ PC + ZrO2	-11.2333(*)	.000	-13.8822	-8.5845
		LPS + PC + Bi2O3	-10.8533(*)	.000	-13.5022	-8.2045
	LPS + MTA	CONTROL	-20.0800(*)	.000	-22.7289	-17.4311
		LPS ALONE	10.5200(*)	.000	7.8711	13.1689
		LPS + PC + CHI3	.0200	1.000	-2.6289	2.6689
		LPS+ PC + ZrO2	-.7133	.938	-3.3622	1.9355
		LPS + PC + Bi2O3	-.3333	.998	-2.9822	2.3155
IL-1 α	CONTROL	LPS ALONE	-24.2067(*)	.000	-26.1096	-22.3038
		LPS + MTA	9.9633(*)	.000	8.0604	11.8662
		LPS + PC + CHI3	9.9300(*)	.000	8.0271	11.8329
		LPS+ PC + ZrO2	9.8900(*)	.000	7.9871	11.7929
		LPS + PC + Bi2O3	9.8300(*)	.000	7.9271	11.7329
	LPS alone	CONTROL	24.2067(*)	.000	22.3038	26.1096

		LPS + MTA	34.1700(*)	.000	32.2671	36.0729
		LPS + PC + CHI3	34.1367(*)	.000	32.2338	36.0396
		LPS+ PC + ZrO2	34.0967(*)	.000	32.1938	35.9996
		LPS + PC + Bi2O3	34.0367(*)	.000	32.1338	35.9396
	LPS + MTA	CONTROL	-9.9633(*)	.000	-11.8662	-8.0604
		LPS ALONE	-34.1700(*)	.000	-36.0729	-32.2671
		LPS + PC + CHI3	-.0333	1.000	-1.9362	1.8696
		LPS+ PC + ZrO2	-.0733	1.000	-1.9762	1.8296
		LPS + PC + Bi2O3	-.1333	1.000	-2.0362	1.7696

- **48 hrs**

Table 32 : Mean +/- SD of OD values expressing mRNA of cytokines at 48 HRS

	CONTROL	LPS alone	LPS + MTA	LPS + PC + CHI3	LPS + PC + ZrO2	LPS + PC+Bi2O3
TNF α	76.21+/- 2.4	13.07+/- 0.7	45.76+/- 2.2	45.62+/- 1.2	46.47+/- 1.9	46.26+/- 0.9
IL-6	65.05+/- 1.6	17.31+/- 1.9	45.03+/- 3.2	46.58+/- 2.2	45.04+/- 0.3	46.70+/- 1.5
IL-1 α	3.50+/- 0.2	41.28+/- 1.7	2.37+/- 0.1	2.41+/- 0.1	2.14+/- 0.4	1.99+/- 0.4

Table 33 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF α	Between Groups	5989.134	5	1197.827	501.848	.000
	Within Groups	28.642	12	2.387		
	Total	6017.776	17			
IL-6	Between Groups	3512.656	5	702.531	180.544	.000
	Within Groups	46.694	12	3.891		
	Total	3559.350	17			
IL-1 α	Between Groups	3767.974	5	753.595	1230.917	.000
	Within Groups	7.347	12	.612		
	Total	3775.321	17			

Table 34 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TNF α	CONTROL	LPS ALONE	63.1400(*)	.000	58.9029	67.3771
		LPS + MTA	30.4500(*)	.000	26.2129	34.6871
		LPS + PC + CHI3	30.5900(*)	.000	26.3529	34.8271
		LPS+ PC + ZrO2	29.7433(*)	.000	25.5063	33.9804
		LPS + PC + Bi2O3	29.9500(*)	.000	25.7129	34.1871
	LPS ALONE	CONTROL	-63.140(*)	.000	-67.3771	-58.9029
		LPS + MTA	-32.690(*)	.000	-36.9271	-28.4529
		LPS + PC + CHI3	-32.550(*)	.000	-36.7871	-28.3129
		LPS+ PC + ZrO2	-33.396(*)	.000	-37.6337	-29.1596
		LPS + PC + Bi2O3	-33.190(*)	.000	-37.4271	-28.9529
	LPS + MTA	CONTROL	-30.450(*)	.000	-34.6871	-26.2129

		LPS ALONE	32.6900(*)	.000	28.4529	36.9271
		LPS + PC + CHI3	.1400	1.00	-4.0971	4.3771
		LPS+ PC + ZrO2	-.7067	.992	-4.9437	3.5304
		LPS + PC + Bi2O3	-.5000	.998	-4.7371	3.7371
IL-6	CONTROL	LPS ALONE	47.7367(*)	.000	42.3267	53.1466
		LPS + MTA	20.0200(*)	.000	14.6100	25.4300
		LPS + PC + CHI3	18.4700(*)	.000	13.0600	23.8800
		LPS+ PC + ZrO2	20.0067(*)	.000	14.5967	25.4166
		LPS + PC + Bi2O3	18.3500(*)	.000	12.9400	23.7600
	LPS ALONE	CONTROL	-47.736(*)	.000	-53.1466	-42.3267
		LPS + MTA	-27.716(*)	.000	-33.1266	-22.3067
		LPS + PC + CHI3	-29.266(*)	.000	-34.6766	-23.8567
		LPS+ PC + ZrO2	-27.730(*)	.000	-33.1400	-22.3200
		LPS + PC + Bi2O3	-29.387(*)	.000	-34.7966	-23.9767
	LPS + MTA	CONTROL	-20.020(*)	.000	-25.4300	-14.6100
		LPS ALONE	27.7167(*)	.000	22.3067	33.1266
		LPS + PC + CHI3	-1.5500	.921	-6.9600	3.8600
		LPS+ PC + ZrO2	-.0133	1.00	-5.4233	5.3966
		LPS + PC + Bi2O3	-1.6700	.896	-7.0800	3.7400
IL-1 α	CONTROL	LPS ALONE	-37.780(*)	.000	-39.9259	-35.6341
		LPS + MTA	1.1300	.517	-1.0159	3.2759
		LPS + PC + CHI3	1.0900	.553	-1.0559	3.2359
		LPS+ PC + ZrO2	1.3667	.331	-.7792	3.5126
		LPS + PC + Bi2O3	1.5160	.239	-.6299	3.6619
	LPS ALONE	CONTROL	37.7800(*)	.000	35.6341	39.9259
		LPS + MTA	38.9100(*)	.000	36.7641	41.0559
		LPS + PC + CHI3	38.8700(*)	.000	36.7241	41.0159
		LPS+ PC + ZrO2	39.1467(*)	.000	37.0008	41.2926
		LPS + PC + Bi2O3	39.2960(*)	.000	37.1501	41.4419
	LPS + MTA	CONTROL	-1.1300	.517	-3.2759	1.0159
		LPS ALONE	-38.910(*)	.000	-41.0559	-36.7641
		LPS + PC + CHI3	-.0400	1.00	-2.1859	2.1059
		LPS+ PC + ZrO2	.2367	.999	-1.9092	2.3826
		LPS + PC + Bi2O3	.3860	.989	-1.7599	2.5319

- **<u>72 hrs</u>**

Table 35 : Mean +/- SD of OD values expressing mRNA of cytokines at 72 HRS

	CONTROL	**LPS alone**	**LPS + MTA**	**LPS + PC + CHI3**	**LPS + PC + ZrO2**	**LPS + PC+Bi2O3**
TNF α	86.28+/- 1.7	8.29+/- 0.5	66.68+/- 1.1	66.69+/- 1.1	65.95+/-3.19	65.99+/- 4.5
IL-6	74.39+/- 1.22	14.88+/- 0.4	64.39+/-1.2	64.52+/- 0.5	65+/- 0.13	65+/- 0.41
IL-1 α	1.29+/- 0.15	51.54+/- 0.8	1.40+/- 0.27	1.4+/- 0.1	1.48+/- 0.28	1.13+/- 0.61

Table 36 : Oneway ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
TNF α	Between Groups	10576.955	5	2115.391	345.560	.000
	Within Groups	73.460	12	6.122		
	Total	10650.414	17			
IL-6	Between Groups	6938.810	5	1387.762	2249.27	.000
	Within Groups	7.404	12	.617		
	Total	6946.213	17			
IL-1 α	Between Groups	6299.477	5	1259.895	5941.50	.000
	Within Groups	2.545	12	.212		
	Total	6302.021	17			

Table 37 : Post Hoc Tests - Multiple Comparisons -Tukey HSD

Dependent Variable	(I) GROUPS	(J) GROUPS	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
TNF α	CONTROL	LPS ALONE	77.9967(*)	.000	71.211	84.7823
		LPS + MTA	19.6067(*)	.000	12.821	26.3923
		LPS + PC + CHI3	19.5967(*)	.000	12.811	26.3823
		LPS+ PC + ZrO2	20.3300(*)	.000	13.544	27.1156
		LPS + PC + Bi2O3	20.2967(*)	.000	13.511	27.0823
	LPS alone	CONTROL	-77.996(*)	.000	-84.78	-71.2111
		LPS + MTA	-58.390(*)	.000	-65.17	-51.6044
		LPS + PC + CHI3	-58.400(*)	.000	-65.18	-51.6144
		LPS+ PC + ZrO2	-57.666(*)	.000	-64.45	-50.8811
		LPS + PC + Bi2O3	-57.700(*)	.000	-64.48	-50.9144
	LPS + MTA	CONTROL	-19.60(*)	.000	-26.39	-12.82
		LPS ALONE	58.3900(*)	.000	51.60	65.1756
		LPS + PC + CHI3	-.0100	1.00	-6.795	6.7756
		LPS+ PC + ZrO2	.7233	.999	-6.06	7.5089
		LPS + PC + Bi2O3	.6900	.999	-6.09	7.4756
IL-6	CONTROL	LPS ALONE	59.5133(*)	.000	57.35	61.6676
		LPS + MTA	9.9967(*)	.000	7.8424	12.1509
		LPS + PC + CHI3	9.8733(*)	.000	7.7191	12.0276
		LPS+ PC + ZrO2	9.1533(*)	.000	6.9991	11.3076
		LPS + PC + Bi2O3	9.3900(*)	.000	7.2358	11.5442
	LPS alone	CONTROL	-59.513(*)	.000	-61.66	-57.3591
		LPS + MTA	-49.516(*)	.000	-51.67	-47.3624
		LPS + PC + CHI3	-49.64(*)	.000	-51.79	-47.4858
		LPS+ PC + ZrO2	-50.36(*)	.000	-52.52	-48.2058
		LPS + PC + Bi2O3	-50.12(*)	.000	-52.27	-47.9691
	LPS + MTA	CONTROL	-9.9967(*)	.000	-12.15	-7.8424
		LPS ALONE	49.5167(*)	.000	47.36	51.6709
		LPS + PC + CHI3	-.1233	1.00	-2.27	2.0309
		LPS+ PC + ZrO2	-.8433	.772	-2.99	1.3109

		LPS + PC + Bi2O3	-.6067	.926	-2.76	1.5476
		LPS + PC + CHI3	.7200	.863	-1.43	2.8742
		LPS + PC + Bi2O3	.2367	.999	-1.91	2.3909
IL-1 α	CONTROL	LPS ALONE	-50.246(*)	.000	-51.50	-48.9838
		LPS + MTA	-.1133	1.00	-1.37	1.1496
		LPS + PC + CHI3	-.1067	1.00	-1.36	1.1562
		LPS+ PC + ZrO2	-.1867	.995	-1.44	1.0762
		LPS + PC + Bi2O3	.1567	.998	-1.10	1.4196
	LPS alone	CONTROL	50.2467(*)	.000	48.98	51.5096
		LPS + MTA	50.1333(*)	.000	48.87	51.3962
		LPS + PC + CHI3	50.1400(*)	.000	48.87	51.4029
		LPS+ PC + ZrO2	50.0600(*)	.000	48.79	51.3229
		LPS + PC + Bi2O3	50.4033(*)	.000	49.14	51.6662
	LPS + MTA	CONTROL	0.1133	1.00	-1.14	1.3762
		LPS ALONE	-50.133(*)	.000	-51.39	-48.8704
		LPS + PC + CHI3	.0067	1.00	-1.25	1.2696
		LPS+ PC + ZrO2	-.0733	1.00	-1.33	1.1896
		LPS + PC + Bi2O3	.2700	.976	-.99	1.5329

Interpretation

The mean +/- SD of all OD values relative to GADPH was obtained and statistical analysis was performed. The results were as follows,

At 24, 48 and 72 hrs,

- Despite LPS stimulation, the qRT-PCR analysis detected moderate to high levels of IL-6 and TNF alpha on all the groups but significantly less than the control group ($p<0.01$).
- IL-1alpha was more detected in control group and significantly increased in LPS alone group ($p<0.01$).
- Despite LPS stimulation, in the presence of experimental material, IL-1alpha expression was minimally detected or it was highly negligible ($p=0$).
- The expression of all cytokines between the experimental groups was statistically insignificant.

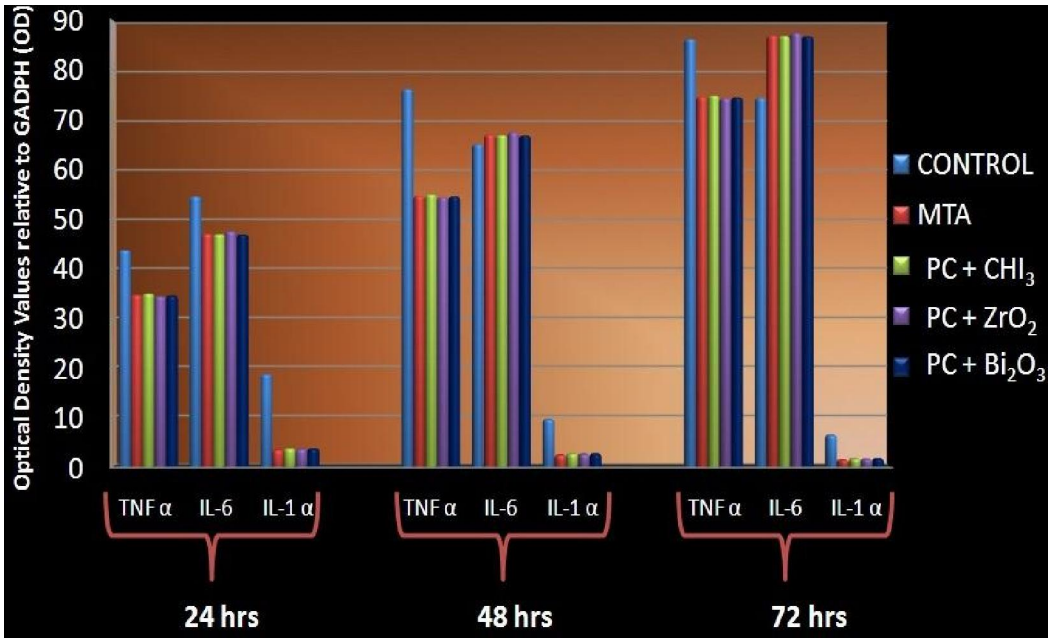


Chart-5 : mRNA expression of cytokines analyzed by qRT-PCR at 24, 48 and 72 hrs.

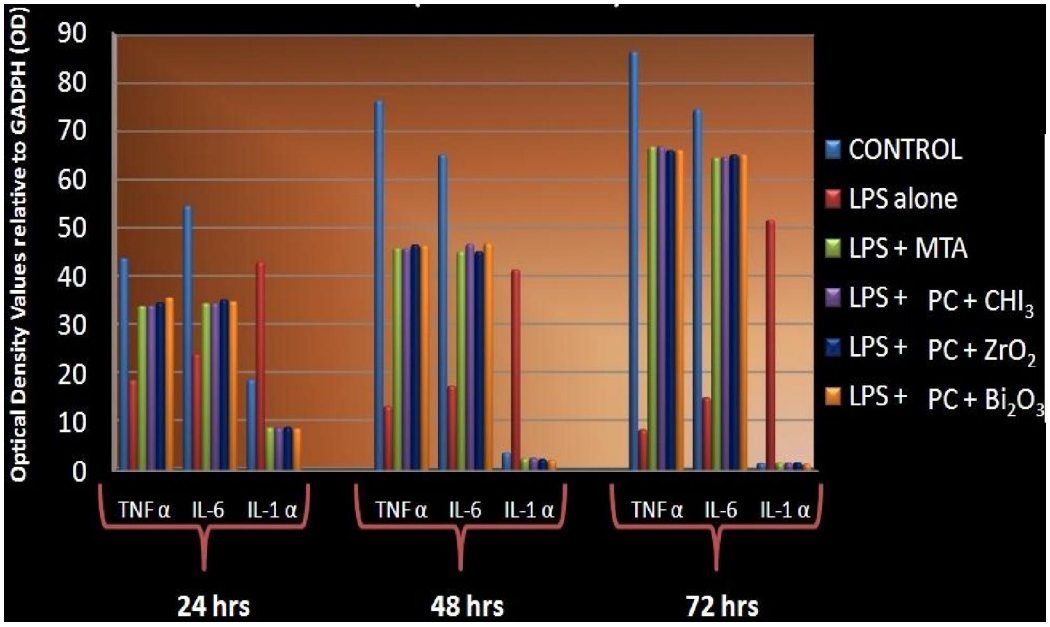
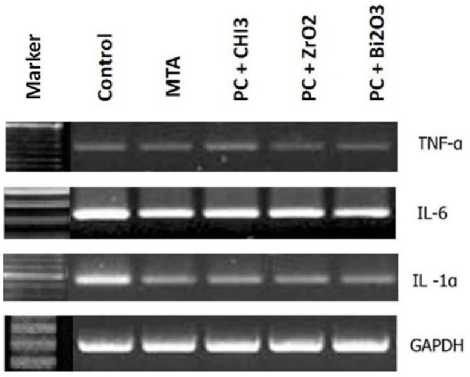
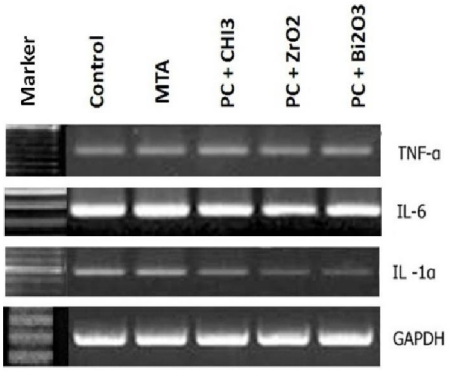


Chart-6 : mRNA expression of cytokines (LPS stimulated) analyzed by qRT-PCR at 24, 48 and 72 hrs.

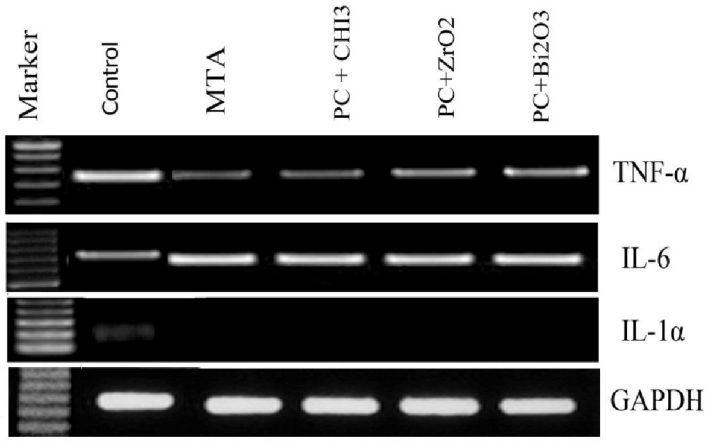
mRNA expression of Cytokines involved in the Mineralization by qRT-PCR analysis at 24, 48 and 72 hrs. GAPDH was used as housekeeping gene.



24 hrs

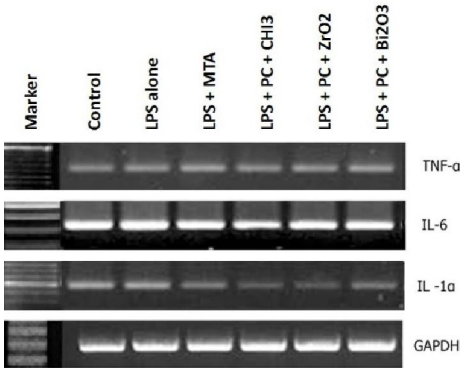


48 hrs

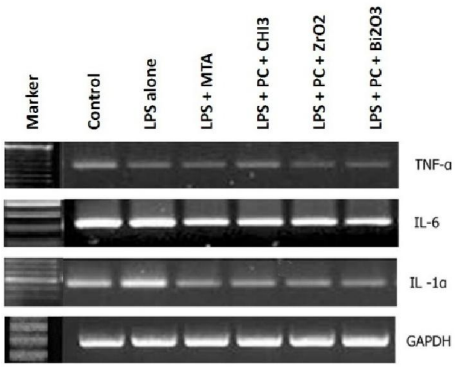


72 hrs

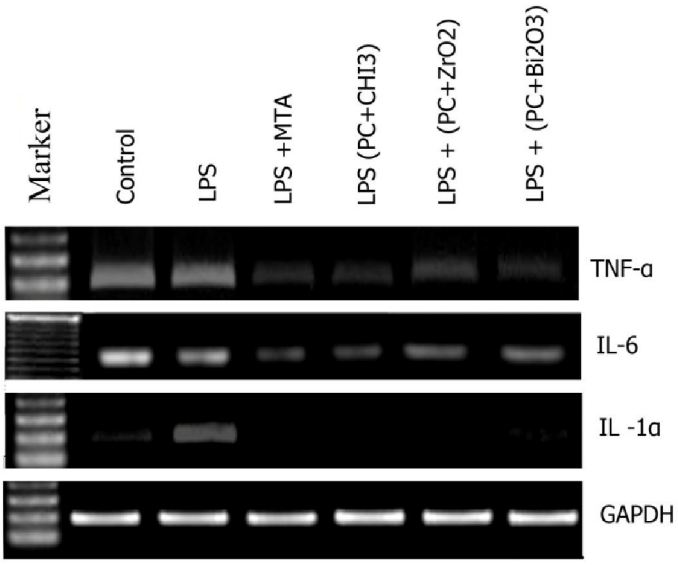
mRNA expression of Cytokines involved in the Mineralization (LPS Stimulated) by qRT-PCR analysis at 24, 48 and 72 hrs. GAPDH was used as housekeeping gene.



24 hrs



48 hrs



72 hrs

Discussion

The growing technology and continuous introduction of new materials for different endodontic applications make the evaluation of the biological properties a mandatory condition. Biocompatibility is the most important property of endodontic materials as because these endodontic materials will be in permanent contact with periapical tissues. Cells in the periapical tissues are mainly Osteoblasts, Cementoblasts and Periodontal ligament cells. Favorable responses to these cells must also be assessed for better evaluation of new endodontic material. Hence Endodontic materials used as furcal perforation repair, root end filling and as an apical plug must be investigated for biocompatibility and other favorable cellular responses to periapical tissues. In the present study we used Pro Root MTA (Dentsply,USA) and white Portland cement – Birla white (Grasim Ind ltd. Aditya Birla group) to compare the favorable cellular responses between them.

Type I Portland cement was present as the main component of MTA. According to manufacturer, Pro Root MTA has 75% Portland cement, 20% Bismuth oxide and 5% dehydrated calcium sulphate. MTA exhibits good in vivo biologic performance when used for root-end fillings, pulp capping and pulpotomy, perforation repairs and apexification treatment[62, 61] MTA promotes biomineralization of osteoblasts and cementoblasts[21] and enhance mineralization[27]. MTA primarily consists of calcium silicate, calcium aluminate, and calcium hydroxide. Ca^{2+} ions are released from hydrated MTA when MTA powder is mixed with sterile water. The amount of Ca^{2+} released has been shown to be adequate for osteoblast survival, proliferation and differentiation[31.] Portland cement is similar to MTA with the exception of radiopacifier, Bismuth oxide which is present only in MTA. Therefore,

calcium ion released from Portland cement may also play an important role in osteoblast cellular responses.

Portland cement is most widely used material in construction. Portland cement is manufactured by clinkering process or partial fusion of raw materials. This process includes lime stone decarbonisation at 400 to 600. It forms dicalcium silicate, tricalcium silicate and tricalcium aluminoferrite between 800 and 1200 c . The tricalcium silicate is formed at 1400 C by the reaction between dicalcium silicate and free lime. Portland cement is classified into different types, depending on the type of additives.[57] White Portland cement (WPC) which is a type I Portland cement contains lower iron content than ordinary Portland cement and therefore it is lighter in color and more esthetic[9].

The chemical components were found to be similar in both Portland cement and Pro Root MTA and there was a small percentile difference. Despite their chemical similarity, difference in the particle texture and size were observed. Pro Root MTA contains 9.2 percentage of bismuth oxide. The X-ray diffraction results showed that tricalcium silicate, dicalcium silicate, tricalcium aluminate and tetracalcium aluminoferrite were the major constitutents of both WPC and Pro Root MTA[25]. The antimicrobial activity was similar in both MTA & WPC and this is due to elevated pH. Torabinejad found that an initial pH of 10.2 for MTA, rising to 12.5 in 3hr. The addition of a radiopacifying agent to Portland cement does not hinder its antimicrobial action[34]. The presence of metals like arsenic in MTA and Portland cement was a major hindrance to their use in medicine. According to ISO 9917-1 standard, water – based cements: Powder/liquid acid base cements (2003), a material to be used in dental procedures should contain no more arsenic than 2 mg/kg of cement. The

concentration of arsenic in Pro Root MTA is 5.25 mg/kg and in white Portland cement is 0.5 mg/kg. MTA is used in small amounts, less than a gram in clinical procedures. Hence 34.27 mg of arsenic per kg of ordinary Portland cement is equal to 34.27µg of arsenic per gm, which is much below the lethal dose of 2 mg/kg of body weight[8].

Lack of radiopacity was a major problem when considering the use of Portland cement as a substitute to Pro Root MTA. Hence Radiopacifying agents should be added to Portland cement. The ISO 6876/2001 standard reported that root canal sealer should be minimum 3 mm Al equivalent radiopaque. According to American National Standard Institute and American Dental Association Sp. No.57, endodontic filling material should present a radiopacity equivalent to 2mm Al in comparison to bone or dentin. Radiopacity of Pro Root MTA was 6.74 mmAl eq and WPC was 0.95 mmAl eq.

Marco Antonio (2009)[32] tested the radiopacity of Portland cement with various radiopacifying agents: Bismuth oxide, Zinc oxide, Iodoform, , Lead oxide, Bismuth carbonate, Barium sulphate, Calcium tungstate and Zirconium oxide. A 20%wt radiopacifiers were added with 80%wt Portland cement. Bismuth oxide, Zirconium dioxide and Iodoform when added with Portland cement had radiopacity values 5.93mmAl, 3.41mm Al and 4.24mmAl respectively. All values are well above the recommended value of ADA/ANSI. In the present study Bismuth oxide, Iodoform and Zirconium dioxide as the radiopacifiers were mixed with the Portland cement. They were mixed with white Portland cement in the ratio of 4:1 (i.e., 20wt% radiopacifying agents + 80wt% WPC). Bismuth oxide was also used as one of the

radiopacifier, so that to compare the response of Portland cement + Bismuth oxide to that of MTA which also contains the same radiopacifier.

Coomarswamy et al (2007) in their study described the effects of bismuth oxide on MTA. He found that the addition of bismuth oxide will decrease the mechanical stability by introducing flaws and increased porosity because of leaving more unreacted water within the Portland cement. This will affect the longevity of the material[14]. Hence there was a need for an alternative radiopacifying agent. Hence we also included Iodoform and Zirconium dioxide as radiopacifying agents in the experimental groups. The Iodoform as a radiopaque agent is added to the WPC because of its good radiopacity, prompt availability and its antiseptic property. Zirconium oxide is added because of its extraordinary properties such as high flexural strength (1,000 MPa), hardness (1,200 – 1,400 VHN) and more esthetic appearance.

The present study has shown that Portland cement with Radiopacifying agents and MTA were similar in terms of supporting a favorable cellular response. The cellular response to these materials has been the subject of investigation. Most of the studies used osteosarcoma cells which resemble osteoblasts. But these osteoblasts alter biological regulatory mechanisms which can lead to an aberrant response[43]. Therefore; other studies used rodent cell lines.[56, 64]. Here in this study we used clonal mouse pre-osteoblastic cell line (MC3T3-E1) because it has high level of differentiation and can express osteoblastic markers such as CbfA1/RunX2, alkaline phosphatase (AP), Bone Sialoprotein (BSP) osteopontin (OPN) and osteocalcin (OCN). Hence it is considered as an excellent model for cellular differentiation studies.

Various methods were used to test biocompatibility of calcium based materials in osteoblastic cell lines. The sample preparation and methodology used were similar

to methodology used in study conducted by **Sema S. Hakki et al. (2009).** According to this method the ratio between the surface of the samples and the volume of medium should be 0.5 cm^2/ml. But in this method, ISO standard (10993-5) was not used. Despite the fact that MTA and other experimental materials was not soluble in water[59], bioactive components from experimental materials might release into surrounding medium and might affect the cellular response in the vicinity of the material. Thus, to determine the effects of factors released by experimental materials on the osteoblasts, a new approach for preparation of experimental materials was carried out in this method. Experimental materials powder was added to MEM containing 5% FCS. At a concentration of 10 mg/ml (10 mg MTA:1 ml MEM), the solution was vortexed until completely mixed and incubated for 24 hours (5% CO2, 37^0C) to extract the bioactive content of the WMTA. Then this extract was used to find the toxicity of the experimental materials at 24, 48 and 72 hrs.

MTA needs wet environment for initial chemical reaction. Hence this method would better mimic the clinical conditions. This technique is also more realistic than the other methodologies because the bioactive components will be released in a different quantity into the surrounding medium before or after setting. Also, the difference in the released bioactive components will interact with surrounding tissues differently.

Sema S. Hakki et al. (2009) reported that 20 mg/ml of experimental material was toxic to cells and hence 10 mg/ml was chosen as an initial concentration and was further diluted into 9 different concentrations. This was done to evaluate the best concentration of the experimental material sample with best cell survival ability and this concentration was used for ALP assay and qRT-PCR analysis. The results

obtained were consistent with results found by **Sema S. Hakki et al. (2009)** and 0.02 mg/ml was the minimum dilution that was chosen as a best experimental material sample.

MTT assay

In the present study, Portland cement with three different radiopacifying agents had no cytotoxicity to MC3T3-E1 osteoblast cells, which was similar to that for MTA. MTT assay measures the metabolic activity of cells grown over the materials under study. Cell proliferation is determined using a redox indicator that can be used to quantitatively measure proliferation of cells. The cell proliferation and cell survival is due to release of Ca^{2+} ions from the experimental materials which maintain the cellular activity at high alkaline pH. In the present study, the cell viability with the presence of all experimental groups was decreased on the first day and second day but increased significantly on the third day compared to the control group. This was due to the initial high pH of the experimental material that inhibited the cell growth on the first and second day. The cell survival ability was statistically insignificant between the experimental groups. This suggests that the Portland cement can be used in place of MTA after addition of any radiopacifier.

ALP assay

This present study tested the ALP activity when experimental materials were in direct contact with osteoblast cells. Alkaline phosphatase activity is an important marker for osteoblastic activity. **Lowry et al (1946)** described the Alkaline phophatase assay. It was done by measuring p-nitrophenol spectrophotometrically. In the presence of alkaline pH, alkaline phosphatase act as an enzyme and can hydrolyze p-nitrophenyl phosphate to yellow colored product, p-nitrophenol. The initial high pH

of the materials and less nutrients may damage the cells and regeneration of cells. This causes reduced ALP activity in day 3. But there is increased osteoblastic differentiation and ALP activity in day 7 and day 15. The osteoblastic differentiation and ALP activity was statistically insignificant between the experimental groups. Hence these materials can be used as root end filling especially in non-vital immature teeth which allows the affected tooth to be restored endodontically after achieving an apical plug. Beyond the apical plug, osteoinductive potential of these experimental materials promotes undisturbed formation of the calcific bridge.

Mineralization associated proteins by qRT-PCR analysis

In this study we evaluated the mRNA expression of mineralization associated proteins (BSP, OPN, OCN AND COL I) and cytokines involved in mineralization (TNF alpha, IL-6 and IL-1 alpha) using quantitative Reverse transcriptase polymerase chain reaction (qRT-PCR). qRT-PCR is the preferred technique to detect and quantify mRNA. The first step is the reverse transcription which synthesizes cDNA from RNA. The second step amplifies cDNA to the detectable levels. This method has been used in various studies,[19,24,49] to detect mRNA expression of osteogenic and odontogenic markers in various cell lines.

BSP, OPN, OCN and COL I are important proteins in mineralized connective tissues. The BSP gene codes for Bone sialoprotein, is highly specific for mineralized tissues like bone and cementum[22.] Highest BSP expression indicates newly synthesized and regenerated bone matrix. The COL1 gene codes for a collagenous protein which is the major component of the extracellular matrix of osteoblasts. Osteocalcin, coded by gene OCN, is a non collagenous protein expressed in the extracellular matrix during osteoblastic mineralization. Osteopontin, coded by gene

OPN, is an organic component of bone and plays an important role in extracellular matrix mineralization[46].

In the present study, we observed at 0.02 mg/ml of experimental materials there is increased induction of osteogenic gene expression (BSP, OPN, OCN, and COL I) from 24 to 72 hrs. The expression level of these four genes was lowest on the first day. It may be due to the pH changes of the experimental materials during setting. When experimental materials were mixed with sterile water, the pH of the medium increased from 7.2 to 12.5 within first 24 hours and this pH change may cause an inhibition on gene expression on day 1[58,54]. But these gene expressions substantially activated by experimental materials on the second day and third day. These gene expressions between the experimental materials were statistically insignificant. This indicates that MTA and Portland cement with radiopacifying agents can induce mineralization and mineralized tissue associated proteins, which play a critical role in bone repair and regeneration, namely osteogenesis. These results emphasize that these materials can be called an osteoconductive and/or osteoinductive material.

Cytokines involved in Mineralization by qRT-PCR analysis

In the presence of osteoinductive /osteoconductive materials like MTA, Osteoblasts form an extensive matrix and express osteogenic phenotypes. However, this favorable response is likely to be modified because of the presence of inflammation and inflammatory agents, like bacterial lipopolysaccharides (LPS) that are available in vivo. Periradicular inflammation is characterized by the cytokines IL-1 alpha, IL-6, TNF alpha, etc.[30.] In particular, IL-1alpha induces most of the bone resorbing activity within periapical lesions.[60] Despite the presence of inflammatory

mediators, materials like MTA have demonstrated remarkable success as root-end fillings. Hence it is well understood that the cellular response to those materials like MTA may involve in modulating inflammation. **Mitchell et al.**[35] found that osteoblast cells produce only IL-6 in response to MTA, without expressing IL-1alpha. **Silva et al.**[53] observed that mouse pulp tissues reduce IL-1 alpha expression, and moderate levels of TNF alpha expression when treated with MTA after pulpotomy. Hence the specific objective was determined in this study whether bacterial LPS–stimulated and unstimulated cells differ in their expression of the inflammatory cytokines IL-1 alpha, IL-6, and TNF alpha.

In this study it was found that TNF alpha and IL-6 are detected in moderate to high levels in all the experimental groups but significantly less than the control group as because experimental materials tend to suppress the inflammatory reaction. IL-1alpha was more detected in control group and its reduction in the presence of the experimental groups was highly significant.

In addition, despite stimulating with Bacterial lipopolysaccharides, it is found that IL-1 alpha was minimally detected in the cultures of all experimental materials. The IL-6 and TNF alpha was found to be in moderate to high levels in all the experimental groups that were treated with bacterial LPS.

Whether the cultures were stimulated with LPS or not, the overall observation of the results obtained was that IL-6 expression was maintained, TNF alpha expression was detected in low levels and IL-1 alpha expression was highly suppressed from 24 to 72 hrs. Osteoblasts produce IL-6 and stimulate osteoclast formation thereby bone resorption. But IL-6 has an additional anti inflammatory activity because of the suppression of IL-1 alpha. Hence these preosteoblasts

maintained IL-6 expressions, whereas their expression of IL-1 alpha appeared to have been suppressed with the presence of experimental material. This favors cessation of IL-1 alpha induced bone resorption in vivo. The expression of all cytokines between the experimental groups was statistically insignificant. This explains that Portland Cement can be used in place of MTA after addition of any radiopacifiers like Bismuth oxide, Iodoform and Zirconium oxide.

Mineral trioxide aggregate (MTA) has been material of choice in several clinical applications. But it is not cost effective and hence its use in all levels of health attention is limited. The present study proved that white Portland cement mixed with radiopacifying agents (Bismuth oxide/Iodoform/Zirconium dioxide) has similar biological responses of MTA. The in vivo experiment in animals (Rat subcutaneous implantation study) was conducted in our department in the year 2010 with the same materials and we found that MTA and Portland cement exhibited similar inflammatory reaction. Hence White Portland cement which is cheaper than MTA can replace MTA in all clinical situations and it will benefit the people at large.

However, further studies like usage tests (Direct pulp capping, Root end filling, Apexification) need to be conducted in vivo in animals and in humans to ensure that this material meets the medical device requirement.

Summary

The aim of the present study was to compare MTA & Portland cement with three different Radiopacifying agents (Iodoform, Zirconium oxide, Bismuth oxide) on Osteoblast cell survival, alkaline phosphatase activity and genetic expression of mineralization.

Materials & Methods

In this study we used clonal mouse pre-osteoblastic cell line (MC3T3-E1) because it has high level of differentiation and can express osteoblastic markers. Mouse osteoblastic Cell lines MC3T3-E1 were obtained from the National centre for cell sciences, Pune. Cells were cultured in Minimal Essential Medium (MEM) supplemented with 10% heat inactivated Fetal bovine serum (FBS), 3% L- glutamine, 100 U/ml penicillin G and 100 µg/ml streptomycin (Hi media) grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

The Portland cement was added with three different radiopacifiers (Bismuth oxide, Iodoform, Zirconium oxide) in the ratio of 4: 1 by weight. Mouse MC3T3-E1 preosteoblasts (100,000 cells per well) were seeded onto 0.02 mg/ml of samples mixed two 24-well tissue culture plates (n=9, 5 groups & 45 wells) for assessing cytotoxicity, alkaline phosphatase assay and for detection of mineralization associated proteins and cytokines by qRT-PCR analysis and three 24-well tissue culture plates (n=9, 6 groups & 54 wells) for detection of LPS stimuated cytokines by qRT-PCR analysis. In case of LPS stimulated culture plates, after 6 hours of growth, fresh medium was added containing 10 micro g/mL LPS in all the groups except group I which serves as a control.

Experimental groups were,

GROUP I - Control

GROUP II - MTA (Pro Root, Dentsply)

GROUP III - White Portland Cement 80wt% + Iodoform 20wt%

GROUP IV - White Portland Cement 80wt% + Zirconium Dioxide 20wt%

GROUP V - White Portland Cement 80wt% + Bismuth Oxide 20wt%

Experimental Groups (LPS stimulated) were,

GROUP I - Control

GROUP II - LPS alone

GROUP III - LPS + MTA

GROUP IV - LPS + White Portland Cement 80wt% + Iodoform 20wt%

GROUP V - LPS + White Portland Cement 80wt% + Bismuth Oxide 20wt%

GROUP VI - LPS + White Portland Cement 80wt% + Bismuth Oxide 20wt%

MTT assay

First, the experimental materials were prepared into 10 different concentrations for assessing cell survival ability using MTT assay. The cell survival ability was compared among the experimental groups at 10 different concentrations and 0.02 mg/ml of the experimental samples was chosen as a best concentration and was used for further comparisons by alkaline phosphatase assay and qRT-PCR analysis. Then the OD values obtained from MTT assay at 0.02 mg/ml of the experimental samples were used to compare between the experimental groups at 24, 48 and 72 hrs.

The cytotoxicty results obtained were statistically insignificant between the experimental groups. The cell viability with the presence of all experimental groups was decreased on the first day and second day but increased significantly on the third

day compared to the control group. This was due to the initial high pH of the experimental material that inhibited the cell growth on the first and second day.

Alkaline Phosphatase assay

Alkaline phosphatase assay was performed at a concentration of 0.02 mg/ml of the experimental samples at 3, 7 and 15 days. The alkaline phosphatase results obtained were statistically insignificant between the experimental groups. At day 3 there was reduced cellular differentiation and ALP activity but at day 7 & and day 15, ALP activity got increased gradually.

mRNA expression of Mineralizaton by qRT-PCR analysis

qRT-PCR analysis was performed for detection of mRNA expression of mineralization associated proteins (BSP, OPN, OCN, and COL I) at 24, 48 and 72 hrs. The results showed that mRNA expression of all mineralization associated proteins was minimal at 24 hrs and 48 hrs but recovered to the level of control group at 72 hrs. The mRNA expression of all mineralization associated proteins was statistically insignificant between the experimental groups.

mRNA expression of Cytokines

The cytokines involved in the mineralization was observed at 24,48 and 72 hrs with and without stimulation with LPS by qRT-PCR analysis and overall observation of the results obtained by qRT-PCR analysis showed that mRNA expression of IL-6 was maintained, TNF alpha was detected in low levels and IL-1 alpha was highly suppressed from 24 to 72 hrs. The expression of all cytokines between the experimental groups was statistically insignificant.

Hence it was concluded that Portland cement can be used in place of MTA after addition of any radiopacifiers like Bismuth oxide, Iodoform and Zirconium oxide.

Conclusion

From the results of the present study, the following conclusions can be arrived,

- Portland cement with three different radiopacifying agents (Iodoform, Zirconium oxide, Bismuth oxide) at a concentration of 0.02 mg/ml was shown to be a nontoxic endodontic material by MTT assay and this cytotoxicity was comparable to MTA.

- The cellular differentiation and alkaline phosphatase activity were increased from day 3 to day 15 for both MTA and Portland cement with three different radiopacifying agents and the results were statistically insignificant between them.

- Both MTA and Portland cement with three different radiopacifying agents expressed mRNA of all mineralization associated proteins (BSP, OPN, OCN, and COL I) and these expressions were statistically insignificant between them.

- With and without stimulating with LPS, both MTA and Portland cement with three different radiopacifying agents modulated inflammation by expressing cytokines TNF α at low levels, IL-1 α at negligible levels and maintaining IL-6 at moderate levels. These modulations were statistically insignificant between them.

- The result from our study proved that Portland cement can be used in clinical situations similar to those in which MTA can be used. Such an inexpensive and easily available material provides successful treatments in all patient population. Nevertheless, further studies are needed to support the clinical applicability of Portland cement.

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